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OBITUARY NOTICES.

FREDERICK WILLIAM PAVY.

Dr Pavy was born at Wroughton in Wiltshire on May 29th, 1829. He received his preliminary education at the Merchant Taylors' School and entered Guy's Hospital in 1847. After five years, he graduated at the University of London, taking the M.B. degree with honours in all subjects and the gold medal in medicine. In the following year he obtained the M.D. degree, and then went to Paris, where he met Claude Bernard, for whom, in after life, he always had the greatest admiration although disagreeing in so pronounced a manner with his teaching. On his return to England he was appointed a lecturer of anatomy at Guy's Hospital in 1854, and lecturer in physiology in 1856, which latter position he held until 1877. In 1859 he became assistant physician at Guy's, and full physician in 1871, when he was also appointed lecturer in medicine at the medical school. On his retirement from active work at Guy's in 1890, he was appointed consulting physician to the Hospital.

Dr Pavy took an active part in the proceedings of the Royal College of Physicians, at which he was sometime examiner, censor and councillor. He was President of the Royal Medical and Chirurgical Society in 1893 and President of the Association for the Advancement of Scientific Research and Chairman of the National Committee of International Medical Congresses until his death on Sept. 19th, 1911. During his career he delivered the Lettsomian Lectures in 1859, the Goulstonian Lectures in 1862, the Croonian Lectures in 1878 and again in 1894, and was Harveian Orator of the Medical Society in 1886. He was elected a Fellow of the Royal Society in 1863.

In 1901 he was awarded the Baly Gold Medal by the Royal College of Physicians for his excellent physiological investigations, and, in celebration of his 80th birthday in 1909, the members of the Physiological Society presented him with a silver bowl in token of their affection for him and admiration of his work. Many other honours were awarded to him not only by his colleagues in this country but by the wider medical fellowship in all parts of the world. He achieved a high reputation for the treatment of diabetes in Europe and America whilst still comparatively young, and his intense vitality enabled him to maintain a commanding position for more than fifty years as an expert in the department which he had made his own.

Although consulted daily by patients from all parts of the world, Dr Pavy was much more than a physician. When the writer was first brought into contact with him towards the end of his active career, he seemed to be the ideal research worker, bending all his energies to search out the solutions of the problems with which he was so familiar. The mornings were given up to consultations, when he was brought into direct contact with the anomalies which reveal the essentially complex nature of the phenomena which are comprised under the term diabetes; the afternoons were devoted to research work, and the evenings to a consideration of the daily results and to the drawing-up of the programme for the next day's experiments. He seemed to be always picturing in his mind the possible sequences of events in the body. Possessed of a remarkably vivid imagination he could *see* the molecules of starchy food being broken down in the alimentary canal into glucose molecules and could watch these simple molecules passing through the walls of the intestines and making their way to the liver, the muscles or the urine. From the visualised processes, deductions were drawn which he sought to substantiate or refute by the test of experiment. He was exceedingly careful, desiring accuracy in his research work and certainty in the deductions to be drawn from it. For him, the positive result of a single experiment was never sufficient to ground a theory on, and he was always on the look-out for the pitfalls which were a cause of stumbling to so many of his co-workers.

The knowledge obtained through long years of experience of the manifold errors by which workers in his field of research were so liable to be led astray impelled him to give but little consideration to contemporary literature. He sought above all else to express his own views in the clearest possible manner, and the only two books he consulted frequently were a large dictionary of several volumes and Roget's *Thesaurus*. The present writer remembers many lengthy discussions over the relative merits of certain kindred words and their ability to express the exact shade of meaning he desired to convey. His writings are models of careful expression and often took weeks and months to prepare. One result of this devotion to accuracy of expression was that he ascribed the same virtue to his contemporaries and often read into their words meanings which the authors themselves did not wish to convey. More than one of the controversies in which he took part arose in this way.

The majority of Dr Pavy's scientific publications are concerned with the metabolism of the carbohydrates in one or other of its aspects. His views on this subject changed very considerably with the passing years. In his early days he discovered that the glycogenic doctrine promulgated by Claude Bernard, which assigns to the liver a regulating function over the supply of carbohydrate to the tissues, was based on faulty experiments. He showed that the saccharine condition of the liver was due to *post-mortem* changes and was absent in the living animal. Further, according to his careful analyses, arterial blood contained only the same amount of dextrose as venous blood, which seemed to prove that dextrose did not pass from the liver to the tissues as free dextrose. Finally he maintained that the kidney acted as a filter which allowed all the diffusible substances in the blood, such as urea and dextrose, to pass into the urine, retaining only the indiffusible proteins within the blood-vessels. "Hence," he argues (in *Carbohydrate Metabolism and Diabetes*, 1906), "if sugar (dextrose) passed as free sugar into the circulation from the food or from the liver, as is contended for under the glycogenic doctrine, the urine could not fail to afford a revelation of the fact." His antagonism to the glycogenic doctrine was thus maintained to the end of his life, although his later experiments convinced him that the glycogen of the liver, after being broken down by enzyme action into dextrose, is normally transported by the blood to the tissues. He could not admit, however, that this passage was in the form of free dextrose, but thought that the dextrose must be adsorbed by, or otherwise loosely combined with, the proteins of the blood.

Dr Pavy regarded the blood as a food of constant composition for the tissue cells. Assimilation occurred at the seat of absorption in the intestines or in the liver, and the work of building-up protein from dextrose and amino-acids was considered by him to be done by the lymphocytes, whilst the transformation of the excess of dextrose into fat and glycogen took place in the columnar epithelium of the mucous membrane of the intestinal wall, and in the liver. Synthesis of protein by the growth of lymphocytes, like yeast cells, on the products of digestion in the wall of the alimentary canal is a view that appeals to the imagination; but it has not met with much favour from the sober-minded physiologists of the present day. The fat-forming powers of the columnar epithelial cells have also been denied, but the exact location of the transformation, which undoubtedly does occur in the body, is still unknown, and it may be that the future will see the vindication of this as well as of other visions of Dr Pavy.

Perhaps the most startling of his discoveries in the domain of biochemistry was the recognition of the carbohydrate groups in the protein molecule. Dr Pavy's theory of the glucosidic nature of the proteins was received with interest throughout the scientific world, and a multiplicity of gluco-proteins have since been isolated. Later investigations have shown, however, that the carbohydrate linkage is not of a glucosidic nature so that the metabolic value assigned to these groups by Dr Pavy has not been generally accepted although no other explanation of their significance has yet been attempted.

Among his contributions to chemical physiology must also be mentioned the method for the quantitative estimation of reducing sugar by means of Pavy's ammoniated cupric test which affords excellent results after a little practice.

In the treatment of diabetes, Dr Pavy did much to provide a more suitable and palatable dietary for the diabetic, and, indeed, was responsible for the introduction of almond and other protein-rich bread substitutes. Regarding excess of free dextrose in the blood of diabetics as exerting a toxic action on the system, it seemed to him essential to stop its flow through the body and his treatment usually commenced by placing the patient on a carbohydrate-free diet. Afterwards, as assimilative power returned, starchy food was allowed, but only in such quantity as to maintain the urine free from dextrose. He was among the first to grasp the importance of the acetone series of substances eliminated especially in acute stages of the disease, and published several papers concerning them. The albuminuria often associated with diabetes naturally interested him as well as the cyclic or physiological albuminuria which he was the first to recognise.

Although many of his views are unorthodox and may be considered improbable, Dr Pavy's critical spirit and his unflagging enthusiasm for experimental research have exerted a stimulating influence on two generations of physiologists, and he has thus been the means, indirectly as well as directly, of extending the borders of our knowledge of carbohydrate metabolism and diabetes. His memory will be cherished by all who were privileged to be brought into intimate relations with him, and his example will be an inspiration throughout all future days.

Dr Pavy married in 1855 Julia, daughter of Mr William Oliver, by whom he had a daughter, Florence, who married the Rev. Borradaile Savory, son of the famous surgeon, Sir William Savory, and father of the present baronet.

H. W. Bywaters.

JOHN WADE.

John Wade, though not a biochemist by title, or indeed by occupation, had the keenest sympathy with all who seek to apply chemistry to the problems of the plant and animal. He was greatly interested in the foundation of the Biochemical Society. During the most active part of his life Wade taught chemistry to medical students, and, unlike some who perform the same duty, he was not content to deal with the subject in a watertight compartment. He had himself no practical knowledge of medicine, but he made it his business to discover, so far as he could, what were the chemical needs of contemporary pathology and clinical medicine, and saw to it that his teaching, in spite of this or that unwise examination schedule, should have some bearing on those needs. He was, it is true, oddly but obstinately convinced that there was something in the nature of biological problems which put them beyond the grasp of his mind, and he was undoubtedly averse from doing any kind of experimental work which did not employ methods of the highest accuracy. Otherwise I think he would have been found among the pioneers of biochemistry, for he was convinced of its outstanding importance. It was perhaps unfortunate for our own particular science that his work was done on other lines, for Wade was a man possessed of great scientific foresight and was a chemist of genius.

He was born at Deptford, on Jan. 13th, 1864. At the early age of 16 he entered the private laboratory of Dr (afterwards Sir Thomas) Stevenson at Guy's Hospital, to act, more or less informally, as a junior assistant. Although this start in life appeared to mark him for a chemist, it was really only long afterwards that Wade definitely chose his career. Not until he was 27 years of age and received the offer of the post of Senior Demonstrator in the Medical School of Guy's Hospital was his decision finally made. Only then did his mind really come to grips with organic chemistry—the subject which he was afterwards to know so intimately and teach so admirably. On his appointment at Guy's he set to work to organise the practical teaching in the new medical school laboratories on original and stimulating lines, and when he felt that this task had been accomplished he began to gather material for his textbook of organic chemistry. During the slow writing of this book, which cost infinite pain and labour, Wade made himself a profoundly learned organic chemist. He went to original sources for every fact, and being gifted with a most retentive memory and an active imagination he stored up an immense amount of knowledge. At the same time his mind was greatly stimulated by contact with the original writings of master chemists. His marked critical power and sense of proportion marshalled the facts of the science into an ordered edifice within his mind, and he became, as I have claimed, really learned in his subject. He produced an admirable book, but, what is more important, the writing of the book made its author an enthusiast[1].

Wade's career was made remarkable by the fact that he was, intellectually, entirely self-made. Few have owed so little to the help of others, or to education in the accepted sense of the word. The only educational scientific lectures he ever attended—save a few connected with evening classes at the Birkbeck Institute—were those he heard as an untaught lecture assistant, and so far as he listened to them at all it was as a youth not yet aware that chemistry was to remain his life's pursuit. He never, at any time of his life, so much as saw a foreign laboratory, and never once shared the responsibility of research work with a senior; never, indeed, saw any research in progress save that inspired by himself. Yet he made his influence deeply felt in the region of science which occupied him, and had he been spared but a few years longer I am convinced that his position among chemists would have been high indeed. He lost his life when his most productive period was beginning. His temperament, his craving for perfection in method and in accomplishment, made the rough adventures of research a trial to him. There were periods, indeed, in his earlier life when he seemed unable to face such adventures, and his output became small; but during the years which immediately preceded his death he acquired great momentum and was producing much. Work, indeed, became nothing short of an obsession with him, and he spared himself so little that those about him became anxious lest he should break down. The interruption to the work came, however, on unexpected and tragic lines, and was, alas, final.

In July, 1912, he was riding a motor cycle when he collided with a cart which had suddenly turned a corner of the road. A shaft struck him violently in the upper part of the chest, fracturing four ribs and damaging a lung. After surviving for three weeks and giving his friends good reason to hope for his ultimate recovery, he died suddenly on August 15th, 1912, as the result of pulmonary embolism.

As Wade's original work dealt with pure chemistry it would be out of place to discuss it here. I have given a moderately full account of it elsewhere[1]. One may point out, however, that in 1902 he published a paper on the constitution of the cyanides which takes classic rank; while from this time onwards he was continuously engaged in a series of extraordinarily careful investigations into the properties of mixtures with a constant boiling point. The choice of this line of research was no accidental matter. Wade was always attracted to the view that atomistic hypotheses are unnecessary to the foundations of chemistry, and believed that all the phenomena are capable of explanation on dynamic lines. He thought that such a dynamical basis would ultimately be found in facts foreshadowed in the properties of "hylotropic" mixtures. How the rehabilitation of the atom by modern physics might have finally affected his views I cannot say. He was always a bold, original and independent thinker.

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[1] Certain paragraphs in this notice are quoted from an account of Wade's life and work written by myself for the Chemical Society, *J. Chem. Soc.* 1913, **103**, 767.

Wade left a widow and one son. The latter, a youth of great promise, obtained last October an entrance scholarship at King's College, Cambridge. After this success he immediately applied for a commission, and is now (Feb. 1916), like other youths of promise, serving his country at the Front.

F. GOWLAND HOPKINS.

CHARLES FREDERICK MYERS-WARD.

C. F. **MYERS-WARD** was born in Birmingham in 1865, and was educated at the High School, New Street; he proceeded afterwards to Queen's College, and Mason College, where he received his medical education. After qualification in 1893 he practised in Birmingham for about two years, and then went to the Owens College, Manchester, as it was then known, to take up the position of Senior Demonstrator of Physiology. A few years later he was elected to the Professorship of Physiology at the University of Sheffield, and while holding that chair was invited to take the Lectureship on Physiology at Charing Cross Hospital Medical School; he accepted this invitation, and his business capacity was soon recognised there by his appointment as the Dean of that School. When Charing Cross a few years ago decided to give up the teaching of the Preliminary and Intermediate subjects of the curriculum, he followed the Charing Cross students to King's College, London, and received the title of Lecturer on

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[1] *J. Chem. Soc.* 1913, 103, 767.

Physiology at that Institution, where he worked successfully and wholeheartedly with Professor Halliburton. He was left in sole charge of the department when the latter left England for a prolonged holiday in 1914, but his occupancy of this position was only a short one, for to the universal regret of all his friends, colleagues and pupils, he died quite suddenly from heart-failure whilst staying at the seaside in August of that year. He married in 1894, Maud, younger daughter of Dr Morris of Birmingham, by whom he had two children, a son and a daughter, who with their mother survive him.

He published papers on "The Structure and Functions of the Mammalian Epididymis," "A Central Chronograph for Physiological Laboratories," and at the time of his death was engaged in a laborious research on Lipase; his results have been incorporated in a paper on that subject written by Dr Umeda and published in this *Journal*.

He devoted his time mainly to making his practical Histology classes as near perfection as possible, and left behind him a unique collection of histological material which King's College was fortunate enough to acquire. He devoted infinite pains to everything he undertook, and as a teacher he was second to none. He had an attractive personality, and not a little of his success was due to the respect and affection he inspired among those associated with him.

W. D. H.

CHARLES GLASS PLAYFAIR LAIDLAW.

C. G. P. **Laidlaw** was born in 1887 in London but he was of pure Scotch descent. He was educated at the Perse School, Cambridge, and at St John's College, Cambridge. In 1912 he obtained a Board of Agriculture Research Scholarship in Plant Physiology. This scholarship he held at the Imperial College of Science and Technology. In the autumn of 1914 he joined the London Scottish regiment. He left England for the front in March, 1915. He was wounded early in April in the trenches at Richebourg l'Avouée and died shortly afterwards; a very promising career was thus cut short. Laidlaw was a very recent member of the Society which he joined only in 1913. The only publication for which he was responsible was one in plant physiology (in collaboration with Mr R. C. Knight) dealing with a new type of recording apparatus for investigating the size of stomata.

V. H. B.

CHARLES MARTIN.

It is with deep regret that we have to record the death of Charles Martin of Abergavenny who was killed in action on May 3rd, 1915, near Ypres.

Martin was educated at Eton. He had not, however, come out in his early days, and he appeared to his contemporaries only as a "modest, simple-minded, rather shy boy, and without the school accomplishments which make boy heroes." He found the lessons boring and got few good reports. Later on he went to Oxford and entered Magdalen College, the President of which was his mother's cousin, and, being a keen field naturalist, he selected Zoology as the subject most likely to interest him. After taking his degree he spent a summer with the Survey of the Freshwater Lochs of Scotland, and published observations on certain Oligochaets and Turbellaria found there. Subsequently he proceeded to the Zoological Station at Naples, and on his return made a brief stay at the Eustace Gurney Laboratory in Norfolk; he was then appointed Demonstrator of Zoology in the University of Glasgow. This post was relinquished in 1911, when he came south, and worked partly at Rothamsted, partly at the Lister Institute, and partly in his own laboratory at his home in Wales.

From the Turbellaria he passed to the Acinetaria, and then to the Protozoa on which most of his work was done. Most of his Papers were published in the *Quarterly Journal of Microscopic Science*; his last two, however, were in the *Philosophical Transactions* and in *The Journal of Agricultural Science* —both on Soil Protozoa.

The great characteristic of Martin's work was the freshness of his outlook. He never would be bound by tradition or convention, or even by time or circumstance. His work, therefore, was really original; he looked at the problem from a new point of view, attacked it in a new way, and always got an interesting result. This was well shown in his work at Rothamsted. He was at once attracted by the hypothesis that soil protozoa constitute one of the factors keeping down the bacterial numbers in the soil, and with characteristic enthusiasm he speedily struck a way through the experimental difficulties with which the subject bristled. He devised a method which for the first time demonstrated the presence of trophic amoebae in the soil and isolated and studied typical organisms present. His naturalist's instincts led him to make some very shrewd faunistic observations, which are being fully confirmed by later work.

A mere discussion of Martin's work, interesting and valuable as it would be, is wholly inadequate to give any picture of what he was like in the Laboratory. In Martin's case you cannot separate the work from the man. While he was with us it was difficult to speak of him without affection, and now that he has gone it is impossible to think of him without emotion. Always boisterously cheerful, never serious, and yet always serious—for he would break off in the midst of the wildest chaff to plunge into the most truculent piece of laboratory work—always uncertain in the minor affairs of life, he was yet firm as a rock in all the things that really matter. You never knew when he would come, how long he would stay, or where he would go next, but you could always know that whether he was here or there he was always the same straight, honest man, free from any taint of unkindness or uncharitableness. And, whether his stay in the Laboratory was long or short, he would bring in a refreshing breeze from the open, and leave behind a wealth of illuminating suggestions.

He was that essentially British product, an amateur in science; a man possessed of private means, a good country house, considerable personal charm and social attainments, moving in a wide circle, and leading a great open life, who from among many possible paths chose that of science because he loved it. So long as we have such men British science can never be without the joy of the enthusiast, and can never degenerate into a mere trade. But just as he refused to be tied down by convention, so he refused to be tied down by science. At Oxford he had run the Magdalen beagles, and at home he was Master of the Crickhowell Harriers, besides fulfilling the other functions of the country gentleman. He had joined the O.T.C. at Glasgow, and when the war broke out went with the Monmouths. It was in the great battle early in May that he was killed by shrapnel while in charge of the machine-gun section. We may be sure that he gave a good account of himself, and that he faced death as fearlessly as he had faced life. It was thus that he would have preferred to go; striking hard and doing something big: dying in defence of those ideals of honour amidst which he had always lived.

E. J. RUSSELL.

ARTHUR CECIL HAMEL ROTHERA.

Arthur Cecil Hamel Rothera was born on July 1st, 1880, at Nottingham. After receiving a sound education at Market Bosworth Grammar School he entered Emmanuel College, Cambridge. I was in close touch with him throughout his University career and from first to last was impressed with his sterling worth and with the high standards of conduct and endeavour which he kept always before him. Even as a youngster fresh from school he took a serious view of his responsibilities: a view indeed which, at the time, was perhaps too serious to be fully understood by his contemporaries. He won friends, however, of the best type, who remained faithful to him and will be faithful to his memory. Hard work, coupled with abilities which were above the average, assured his success at Cambridge. He obtained a first class in the Natural Science Tripos Part 1, in June, 1902, and in the following year a first class in Part 2, his subject being physiology.

He was early attracted towards the chemical side of physiological studies, and after obtaining his degree he came to tell me that he would look to biochemistry for a career. As a result of this decision, which at that time called for some courage, he determined to obtain a medical qualification as quickly as possible and then return to Cambridge for research in his chosen subject. He went to St Mary's Hospital and in 1904, after having performed the duties of some of the essential clinical appointments, but, as yet, unqualified, he came back to the University and spent some months upon a study of cystine and sulphur metabolism. During the same year he went to Strassburg and carried out a brief research in the domain of protein chemistry under Hofmeister. Later he returned to hospital for further medical work while occasionally visiting Cambridge to obtain laboratory experience.

In the year 1906, as the result of the influence of Professor W. A. Osborne, a lectureship in biochemistry was created in the University of Melbourne.

As a member of a small committee which was asked to select candidates for this post I felt justified in pressing Rothera's claims and he was eventually appointed to it. The opportunity came rather early in his career. He was young, somewhat shy and reserved, and he possessed, of course, no great experience either of teaching or research. To place him in so important a post, one of the very first to be definitely associated with the name of the academically new subject of biochemistry, was certainly of the nature of an experiment. It is therefore a source of keen pleasure to those who had faith in him to read the words of Professor Osborne, his senior colleague at Melbourne, written after Rothera's nine years of service and untimely death.

"Dr Rothera," writes Professor Osborne, "was a biochemist of the best type....With all those varying activities with which biochemistry comes into contact he was in the closest sympathy. The medical student had the value of his teaching in the physiology class room, and in the lectures on pharmacology, but he had little opportunity of realising the splendid service which was rendered to agriculture, not only in the agricultural biochemistry course which Dr Rothera created here, and which inspired every pupil who took up the subject, but in special research work for the Agricultural Department. To-day those who have the future of scientific agriculture at heart are deploring with us the loss of a highly skilled adviser and investigator who we fear cannot easily be replaced." Professor Osborne says further, "Any estimate of Dr Rothera's life work which was restricted to his publications and purely professional duties would be very inadequate. For he influenced pupils and friends by the charm of his personality, by the integrity of his character and by the inexhaustible loyalty of his friendship." This is high praise from one who was in close touch with the man he is describing.

I am tempted to recall the circumstances of Rothera's preparation for his first journey to Melbourne as they illustrate his grit. The appointment came somewhat unexpectedly, and he was left with a very brief period—I believe it was a fortnight only—to wind up his affairs in this country, to get his equipment together, to sit for the final examination of the Conjoint Board, and, above all, to cross the Channel and persuade the family of the charming French lady who was to be his wife to consent to an arrangement which would later on involve her joining her husband in a country most remote. All was successfully accomplished in the brief period mentioned and Rothera sailed to begin a career which though it was to prove lamentably short was to bring him satisfaction and happiness.

After the war began I received letters from him which showed unrest of mind. He was unable to settle to any work which did not involve direct service to his country in her need. In August, 1915, he broke away and with the consent of the University he obtained a Captaincy in the R.A.M.C.: but the service he longed to give was unhappily soon terminated. He contracted influenza while working in camp, and a fatal attack of pneumonia led to his death on Sunday, Oct. 3rd, 1915. He died with his scientific work little more than begun, but the subject of his choice has benefited from his labours and his colleagues and successors will not forget what he accomplished.

The circumstances of his death were certainly most sad and lent more than ordinary pathos to the loss suffered by his widow and her two young children. Yet though his career was short and his end untimely it must not be forgotten that his work had always been such as he loved and enjoyed.

In 1905 Rothera published a paper dealing with a modification of the nitroprusside test for acetone which has proved extremely useful. As Professor Osborne remarks, one can say without exaggeration that it is used in practically every medical school and hospital throughout the world. Rothera soon realised, when he found himself at work in Melbourne, that in a new country the University teacher must give attention to practical problems. Much of his time and energy were expended upon a study of the chemistry and physiology of milk. These studies bore upon the needs of local industries but they also possessed scientific value. Some of this work was published, but much of it may now never see the light of publication. His last paper (with Mr L. A. L. Maxwell) described experimental results, which must have been very difficult to obtain, in proof of his belief that the action of pituitary extracts in promoting the flow of milk is the result of a true secretory stimulus and not merely due to muscular contractions.

F. GOWLAND HOPKINS.

I. THE FATE OF INORGANIC NITROGEN IN THE METABOLISM OF THE DOG.

By WILLIAM CALDWELL
and HAROLD REX SEPTIMUS CLOTWORTHY.

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These experiments were begun in January, 1913, and were undertaken with the object of confirming or otherwise the generally accepted view which prevailed at that time, namely, that retention of nitrogen takes place when the proteins of the food are replaced by inorganic nitrogen in the form of ammonium salts. As the investigation—the results of which are contained in Table I—proceeded, it was thought that it would be an interesting addition to the work to see in what forms the nitrogen was excreted, and if it was retained, to determine whether the retention was temporary or permanent.

The meaning of the expression "retention of nitrogen" is somewhat ambiguous. If the weight of the animal remains constant or increases, and some or all of the extra nitrogen given in the food or injected into the body is retained at the same time, then undoubtedly nitrogen is retained. On the other hand, if more nitrogen is excreted than is given with the food or injected into the body, although the weight of the animal increases, it is assumed that no nitrogen is retained. Again, if the weight of the animal decreases, and some of the extra nitrogen given is not immediately excreted, it is assumed that the decrease is due to a loss of water, and that in this case there is retention of nitrogen, though it may be temporary.

Peschek [1911] was the first to call attention to this problem. He fed an animal on food which contained a high percentage of nitrogen, and added ammonium acetate for certain periods to the food. His results, however, were contradictory—sometimes nitrogen was retained, and at other times it was lost.

The problem has since been investigated by a number of authors. Grafe and Schlaepfer [1912] assert that an animal has the power to retain nitrogen and use it to synthesise protein from nitrogen-free food (carbohydrates and fats) and inorganic nitrogen in the form of ammonium salts. Abderhalden [1912] also came to the conclusion that clear evidence was obtained of the formation of protein, when dogs were fed on a nitrogen-free food with the addition of inorganic nitrogen. But on an examination of his results it will be seen that in the first experiment there is a negative nitrogen balance and a continual loss in weight. Similarly in Exp. 2 there is a negative nitrogen balance and a continual loss in weight. In Exp. 3 a dog which had lost 2000 g. recovered its original weight rapidly—in 11 days—by feeding with the hydrolysed products obtained from flesh and blood. The dog was then fed on a nitrogen-free diet, and the animal lost weight, and the nitrogen balance was negative during this part of the experiment. Then ammonium acetate was added to the carbohydrate and fat food. The nitrogen balance was positive for five days and negative for the remainder of the period. The result of the feeding for the whole period was a retention of nitrogen and a slow but gradual decline in weight. In the last period of this experiment the amount of ammonium acetate given with the carbohydrates and fats was increased but the nitrogen balance remained negative during the whole period—5·34 g. nitrogen being lost—and the weight fell gradually but slowly. In Exp. 4 the nitrogen balance was negative during the whole of the experiment. Thus what Abderhalden has proved, apart from confirming the established fact, that the protein of the food may be replaced by the completely hydrolysed products obtained from flesh and blood, is, that the addition of inorganic nitrogen to a nitrogen-free food prevents the rapid loss of weight which takes place when an animal is fed on carbohydrates and fats alone. His experiments do not conclusively show a permanent retention of inorganic nitrogen and Abderhalden has lately admitted this. At the end of a long investigation [Abderhalden, 1913], he states that ammonium salts, and especially ammonium acetate, can influence at times the nitrogen balance in the sense of a retention, but, he adds, there are no grounds, from the results which he has obtained, for the assumption that protein can be formed from ammonia and nitrogen-free compounds, such as carbohydrates and fats. More recently the problem of the retention of nitrogen has been investigated by Taylor and Ringer [1913] who find that the presence of carbohydrates in the food is not a necessary factor in the retention of nitrogen from ammonium salts; and also by Underhill [1913, 1], whose communications are important. He fed a dog on an adequate mixed diet. The ammonium salts of organic acids were given to the dog orally, and it was found that none of the added nitrogen was excreted as ammonia nitrogen. When however the ammonium salts of inorganic acids were given to the dog, the added nitrogen was excreted partly as ammonia nitrogen—with ammonium chloride 52 % of the added nitrogen, and with ammonium phosphate 64 % of the added nitrogen appeared in the urine. In a later paper Underhill [1913, 2] contests the conclusions of Grafe and Schlaepfer whose work he repeats but fails to find evidence of any of the inorganic nitrogen being used in the synthesis of proteins.

The analogy between the plant cell and the animal cell does not seem to hold in respect to nitrogen; for, while syntheses are not denied to the animal cell, it has not yet been shown that it is able to synthesise complex organic substances from inorganic constituents. Again, we are much inclined to discuss results as if an animal were a perfect mechanical balance, which it is not; for, whilst approximate nitrogenous equilibrium can be attained, absolute nitrogenous equilibrium can scarcely be said to exist. A fair margin has to be allowed for temporary fluctuations on either side of the line of absolute equilibrium.

METHOD.

We fed a dog on a fixed diet containing a definite amount of nitrogen as determined by analysis, for a considerable time before the actual experiment began, to enable the dog to acquire a steady state or condition in which the amounts of nitrogen excreted per day should be approximately equal. When this steady state had been attained a known amount of an ammonium salt was added to the food—one-half to the food in the morning, and the remaining half to the food in the evening. This was continued for three or four days. An interval of three or four days was then allowed to elapse before a second ammonium salt was added to the food, and so on. Our feeding periods therefore extended for three or four days and the intervals after the addition of the extra nitrogen also extended for three or four days. The bladder was emptied regularly by a sterilised catheter at intervals of 24 hours. The day's urine was measured and a complete analysis was performed each day, whether during a period or an interval. The faeces were collected and grouped into periods corresponding to the experimental and the interval periods.

The amount of water which the dog drank was accurately recorded.

In some of the experiments the ammonium salt was injected subcutaneously in sterile condition—half of the volume was injected in the morning after food, and the other half injected in the evening after food.

These injections were made for us by Professor W. H. Thompson.

The diet consisted of 100 g. of potatoes, 100 g. dog biscuit, 50 g. flour, 10 g. dried milk, 10 g. lard, 2 g. salt and 250 g. water.

Dog XV.

The results obtained with dog XV are shown in Table I.

The investigation of the results of feeding with ammonium salts had been in progress for some time before analyses to ascertain the manner in which the extra nitrogen was excreted were undertaken. As a consequence the details of the early part of the experiment are in this respect not complete.

Period III. 0·617 g. nitrogen was added to the food as ammonium chloride and 0·939 g. appeared—0·312 g. or 33·2 % as ammonia nitrogen and 0·458 g. in the faeces.

Though there was a positive balance, yet compared with period II there was a loss of nitrogen.

Period IV. During this interval the amount of ammonia nitrogen decreased and became almost equal to the amount of ammonia nitrogen during period II. The period was a long one and may be compared with the succeeding feeding period.

Period V. 0·413 g. nitrogen was added to the food as ammonium carbonate. It will be seen that the amount of ammonia nitrogen has not been increased by the addition of ammonium carbonate to the food.

Period VI. The amount of ammonia nitrogen remained approximately steady.

Period VII. 0·315 g. nitrogen was added to the food as ammonium phosphate; more than this amount of nitrogen appeared, as may be seen on comparing the results with period VI or with period II. The amount of ammonia nitrogen was increased by 0·331 g.—an amount greater than the amount of nitrogen added.

Period VIII. During this interval the amount of ammonia nitrogen again became approximately normal, but on the addition of sodium phosphate to the food, the amount of ammonia nitrogen excreted was decreased, and the amount of urea nitrogen increased.

From this investigation it would appear therefore that the amount of ammonia nitrogen excreted depends on the nature of the ammonium salt

TABLE I. Dog XV.

Period	Weight g.	Nitrogen given per day g.	Nitrogen excreted per day g.	Nitrogen balance g.	Nitrogen added g.	Nitrogen excreted g.	Urea nitrogen g.	Ammonia nitrogen g.	Creatinine mg.	Creatine mg.	Nitrogen in faeces g.
I. 6 days. Ammonium acetate (in food)	7,003	4·369	3·786	0·583 +	0·539	—	—	0·0726	192·9	44·7	0·942
II. 8 days. Interval ...	7,048	3·830	2·844	0·986 +	—	—	—	0·0749	199·8	43·7	0·317
III. 6 days. Ammonium chloride (in food)	7,156	4·447	3·783	0·664 +	0·617	0·939	—	0·3869	207·8	24·8	0·775
IV. 8 days. Interval ...	7,282	3·830	3·163	0·667 +	—	0·319	—	0·0847	208·0	26·8	0·634
V. 6 days. Ammonium carbonate (in food)	7,424	4·243	3·542	0·701 +	0·413	0·698 (0·379)	—	0·0737	197·1	25·3	0·713
VI. 3 days. Interval ...	7,535	3·830	3·562	0·268 +	—	0·718	—	0·0676	195·1	20·3	0·697
VII. 6 days. Ammonium phosphate (in food)	7,609	4·145	4·040	0·105 +	0·315	1·196 (0·478)	2·3599	0·3981	204·5	45·3	0·731
VIII. 3 days. Interval	7,779	3·830	3·490	0·340 +	—	0·646	1·756	0·0733	199·4	20·7	0·786
IX. 5 days. Sodium phosphate (in food)	7,824	3·830	3·620	0·210 +	—	0·776	2·153	0·0366	208·6	25·1	0·855
X. 3 days Interval ...	7,929	3·830	3·524	0·306 +	—	0·680	1·977	0·0550	197·3	26·6	0·752

added to the food. The ions into which the ammonium salts are dissociated may or may not act as foreign substances in the body. In the case of ammonium carbonate, the body does not find it necessary to protect itself but in the cases of ammonium chloride and ammonium phosphate it would seem that the decomposition of the ammonium salt demands from the body some kind of protection from the hydrochloric acid or the phosphoric acid, and the ammonia produced is excreted; hence the increase in the amount of excreted ammonia nitrogen.

The addition of sodium phosphate to the food is somewhat interesting. Di-sodium hydrogen phosphate is slightly hydrolysed in solution and has an alkaline reaction. The effect of such a substance would seem to decrease the amount of ammonia nitrogen excreted and increase the amount of urea nitrogen excreted.

The conclusion we may legitimately draw from the foregoing investigation is that an ammonium salt of a weak acid has little or no effect on the amount of ammonia nitrogen excreted; whereas the ammonium salt of a strong acid increases very considerably the amount of excreted ammonia nitrogen.

While the nitrogen balance was positive during the whole experiment, the amount of nitrogen excreted increased during the course of the experiment. There was therefore no retention of the added nitrogen during this series of experiments.

DOG XVII. (TABLE II.)

Dogs XV and XVIII were reserved exclusively for the investigation of the metabolism of the ammonium salts mentioned in the tables which record the results. These dogs were kept for a very considerable time on a constant diet before the real investigation began. Dog XVII was not treated in such a systematic manner.

In this investigation the ammonium salts of lactic acid and citric acid were used. When the salts were injected there was not a very marked increase in the amount of the ammonia nitrogen excreted. The amount of urea nitrogen excreted increased when ammonium lactate was injected and decreased when ammonium citrate was injected. The behaviour of these salts on injection, however, is in strange contrast to the behaviour of the same salts when they are added to the food.

During period IV, 0·550 g. nitrogen was added to the food as ammonium lactate, and 0·480 g. nitrogen appeared as excreted nitrogen. Of this excreted nitrogen 0·436 g. or 90·8 % appeared as urea nitrogen. During period VI

TABLE II. DOG XVII.

Period	Weight g.	Nitrogen given per day g.	Nitrogen excreted per day g.	Nitrogen balance g.	Nitrogen added g.	Nitrogen excreted g.	Urea nitrogen g.	Ammonia nitrogen g.	Creatinine mg.	Creatine mg.	Nitrogen in faeces g.
I. 5 days. Interval ...	8,208	3·83	3·908	0·078 –	—	—	2·615	0·155	261	12	0·875
II. 5 days. Ammonium lactate (injection)	8,293	4·09	4·326	0·236 –	0·260	0·418	2·707	0·178	264	20	0·9298
III. 4 days. Interval ...	8,341	3·83	3·504	0·326 +	—	—	2·371	0·163	233	41	0·643
IV. 5 days. Ammonium lactate (in food)	8,372	4·38	3·984	0·396 +	0·550	0·480	2·807	0·148	240	58	0·656
V. 3 days. Interval ...	8,419	3·83	3·905	0·075 –	—	—	2·465	0·148	232	55	0·920
VI. 5 days. Ammonium citrate (in food)	8,494	4·38	4·283	0·097 +	0·550	0·378	2·741	0·144	234	49	0·833
VII. 3 days. Interval ...	8,548	3·83	3·660	0·170 +	—	—	2·381	0·132	239	49	0·777
VIII. 3 days. Ammonium citrate (injection)	8,561	4·10	3·996	0·104 +	0·270	0·336	2·308	0·168	242	51	0·931
IX. 3 days. Interval ...	8,590	3·83	3·685	0·145 +	—	—	2·393	0·146	238	53	0·816

0·378 g. nitrogen appeared as excreted nitrogen after the addition of 0·550 g. nitrogen to the food as ammonium citrate, and of this excreted nitrogen 0·276 g. or 73 % appeared as urea nitrogen.

Dog XVIII. (Table III.)

Period I. The steady state is represented by:—3·830 g. nitrogen given per day, and 3·545 g. nitrogen excreted per day.

Period II. 0·47 g. nitrogen was added to the food as ammonium acetate, and of this 0·424 g. appeared as excreted nitrogen; 0·383 g. or 90·3 % of this excreted nitrogen was in the form of urea nitrogen.

0·046 g. nitrogen was retained during the period, but this amount was lost in the succeeding interval. Not only so, but a loss of 0·914 g. nitrogen occurred in interval III and this nitrogen was excreted in the faeces of interval III. The added nitrogen therefore seems not only to be excreted, but to have caused some decomposition of nitrogenous substances. The result of feeding with ammonium acetate is therefore an unaccountable loss of nitrogen. It may be a washing out of temporarily retained nitrogen.

There was a decrease of 100 g. in the weight of the dog during the interval III.

Period IV. 0·283 g. nitrogen was injected as ammonium acetate, and 0·675 g. nitrogen appeared as excreted nitrogen. 0·443 g. or 60·6 % of this excreted nitrogen appeared as urea nitrogen, and 0·12 g. nitrogen appeared as excess in the faeces. There was thus a loss of nitrogen during the period.

Period V. During this interval there was a very slight loss of nitrogen compared with the state of equilibrium which existed at the beginning of the experiment.

Period VI. 0·61 g. nitrogen was added to the food as ammonium chloride and of this 0·490 g. appeared as excreted nitrogen. In comparison with the "steady state" at the beginning of the experiment, 0·297 g. or 60·5 % was excreted as urea nitrogen, and 0·327 g. or 66·7 % as ammonia nitrogen. But in comparison with interval V, 0·135 g. or 27·5 % appeared as urea nitrogen, and 0·354 g. or 72·2 % as ammonia nitrogen.

0·12 g. nitrogen was retained at the end of the period.

Period VII. During this interval not only was the retained nitrogen lost, but 0·416 g. extra nitrogen was excreted, principally in the faeces. The urea nitrogen excreted was 0·202 g. less than normal, whilst the nitrogen in the faeces was 0·67 g. more than in the normal condition. Most of this extra excreted nitrogen appeared on the day following the previous period.

TABLE III. Dog XVIII.

Period	Weight g.	Nitrogen given per day g.	Nitrogen excreted per day g.	Nitrogen balance g.	Nitrogen added g.	Nitrogen excreted g.	Urea nitrogen g.	Ammonia nitrogen g.	Creatinine mg.	Creatine mg.	Nitrogen excreted in faeces g.
I. 5 days. Interval ...	6,735	3·830	3·545	0·285 +	—	—	2·2861	0·1919	176·76	68·02	0·790
II. 5 days. Ammonium acetate (in food)	6,887	4·300	3·969	0·331 +	0·47	0·424	2·6692	0·1878	175·62	96·10	0·7878
III. 4 days. Interval ...	6,787	3·830	4·459	0·629 –	—	0·914	2·2673	0·1857	176·85	38·15	1·741
IV. 3 days. Ammonium acetate (injected) ...	6,837	4·113	4·220	0·107 –	0·283	0·675	2·7292	0·1608	182·6	21·3	0·912
V. 3 days. Interval ...	6,771	3·830	3·576	0·254 +	—	0·031	2·4482	0·1658	170·6	26·4	0·9037
VI. 5 days. Ammonium chloride (in food)	6,830	4·440	4·035	0·405 +	0·61	0·490	2·5834	0·5190	181·2	32·1	0·825
VII. 3 days. Interval ...	7,016	3·830	3·961	0·131 –	—	0·416	2·0849	0·2081	177·8	21·0	1·461
VIII. 5 days. Ammonium chloride (injected)	6,980	4·418	4·217	0·201 +	0·588	0·672	2·4746	0·4150	189·8	27·7	0·855
IX. 2 days. Interval ...	6,938	3·830	3·075	0·755 +	—	0·470 (Retained)	2·3040	0·1925	184·7	78·8	0·3495
X. 3 days. Ammonium phosphate (in food)	7,107	4·281	4·210	0·071 +	0·4508	0·665	2·2941	0·3989	191·6	32·0	1·267
XI. 2 days. Interval ...	7,122	3·830	3·635	0·195 +	—	0·090	2·0741	0·2429	192·7	27·1	1·063
XII. 3 days. Ammonium phosphate (injected)	7,268	4·334	3·680	0·654 +	0·504	0·135	2·4377	0·3103	203·4	40·3	0·584
XIII. 2 days. Interval ...	—	3·830	—	—	—	—	2·0594	0·3136	179·3	71·9	—

Period VIII. 0·588 g. nitrogen was injected as ammonium chloride, and 0·672 g. nitrogen appeared as excreted nitrogen. In comparison with the "steady state" 0·1885 g. or 28 % appeared as urea nitrogen, and 0·2231 g. or 33·2 % as ammonia nitrogen; whereas in comparison with the previous period 0·3897 g. or 58 % appeared as urea nitrogen, and 0·2069 g. as ammonia nitrogen—an amount very slightly in excess of the added nitrogen injected.

Period IX. During this interval a comparatively large amount of nitrogen was retained from the regular food, 0·755 g. in comparison with 0·285 g. during the equilibrium stage at the beginning of the experiment. The amount of urea nitrogen became normal, and so also did the ammonia nitrogen, whilst the amount of nitrogen excreted in the faeces was 0·45 g. less than during the first period.

Period X. 0·4508 g. nitrogen was added to the food as ammonium phosphate, and 0·665 g. appeared as excreted nitrogen. The urea nitrogen excreted remained normal, but the ammonia nitrogen was 0·207 g. (or 31 % of the excreted nitrogen) in excess of that of the equilibrium stage. 0·47 g. nitrogen was excreted in the faeces more than the normal amount. This amount and the amount of extra urea nitrogen excreted make up the total amount excreted.

Period XI. During this interval some nitrogen was lost—0·09 g. The urea nitrogen excreted was 0·22 g. less than in the normal state, but against that the faeces contained 0·27 g. nitrogen in excess of that of the first period.

Period XII. 0·504 g. nitrogen was injected as ammonium phosphate and 0·135 g. appeared as excreted nitrogen. 0·15 g. appeared as urea nitrogen, and 0·12 g. as ammonia nitrogen, whilst 0·21 g. nitrogen less than the normal amount was excreted in the faeces. In this period a very considerable amount of nitrogen is retained.

It was noted during the experiment that the amount of ammonia nitrogen varied directly as the acidity of the urine, determined by titration with N/10 alkali, using phenolphthalein as indicator. The urine itself, as was to be expected, was never alkaline to phenolphthalein. The exact determinations will be published in a later paper.

An interesting point is to compare the periods of feeding with the periods of injection in the foregoing experiments.

1. The experiments with ammonium chloride (Table III, periods V–VIII). On comparing the interval immediately before the feeding experiment with the feeding period itself, it will be seen that the extra nitrogen which was excreted appeared as 0·135 g. or 27·5 % urea nitrogen, and 0·354 g.

or 72·2 % ammonia nitrogen, whereas in the case of the injection of ammonium chloride, the extra nitrogen which was excreted appeared as 0·3897 g. or 58 % urea nitrogen, and 0·2069 g. or 30·8 % ammonia nitrogen.

The injection seems to reverse the magnitude of the amounts of excreted nitrogen as urea nitrogen and as ammonia nitrogen respectively, when compared with the feeding experiment.

2. The experiments with ammonium phosphate (Table III, periods IX–XIII). On comparing the interval immediately preceding the feeding experiment with the feeding experiment period, it will be noticed that the extra excreted nitrogen appears as ammonia nitrogen only, but in the case of the injected ammonium phosphate, the extra excreted nitrogen appears as 0·36 g. urea nitrogen and 0·07 g. ammonia nitrogen, a reversal of magnitude similar to the experiments with ammonium chloride.

3. The experiments with ammonium acetate (Table III, periods I–IV). On comparing the feeding experiment with the interval immediately preceding the feeding experiment, it will be seen that the extra excreted nitrogen appears as urea nitrogen only. The ammonia nitrogen is almost identical with that of the previous period. Similarly with the injected ammonium acetate. The extra excreted nitrogen appears as urea nitrogen only, but the ammonia nitrogen is 0·025 g. less than the previous period.

There is no such marked reversal as that noted in the cases of ammonium chloride and ammonium phosphate.

Taking the experiment as a whole there was no retention of the added nitrogen. The animal was excreting more nitrogen at the end of the experiment than during the "steady state" period; yet the weight of the animal had increased by more than 500 g. during the investigation, which lasted about nine weeks.

The health of the animal during the experiment was excellent.

DISCUSSION AND SUMMARY.

Speculations concerning the synthetic powers of the body under conditions such as we have been investigating are very attractive, but we hesitate to make positive assertions. Even when the extra nitrogen is given in the form of ammonium salts, and it appears as urea, we hesitate to say that the ammonia nitrogen is synthesised into urea. It is no doubt true that the ammonium salt is dissociated. That is evident when we consider the feeding experiments with ammonium chloride. The dissociation of the ammonium chloride into ammonia and hydrochloric acid and the separation of these products involve a kind of protection on the part of the body, and the formation of a large amount of ammonia, which is subsequently excreted, to protect itself against the acidity of the hydrochloric acid. If the urea is produced from ammonia and carbon dioxide it will involve not only the dissociation of the ammonium chloride, but the oxidation of the ammonia and the reduction of the carbon dioxide in a fashion which we shall not discuss. We do not suggest that the nitrogen is not made use of in synthesis or replacement. It may be, though, that the ammonium salts have some other action, such as displacement, with decomposition of the substances which produce urea.

The chief interest in our work is the manner in which the extra nitrogen taken with the food or injected into the body is excreted. The form in which it is excreted depends on the ammonium salt employed, and apparently on the ions into which the ammonium salt is dissociated. For instance, the added nitrogen in the case of ammonium acetate is excreted in the form of urea. In the case of ammonium chloride and ammonium phosphate, it is excreted both as urea and ammonia. The acetic acid derived from ammonium acetate is a much weaker acid than the hydrochloric acid, or the phosphoric acid derived from the ammonium chloride and ammonium phosphate respectively. The body does not find it necessary to protect itself by the production of ammonia, to neutralise the acetic acid, but it is compelled to protect itself in the case of hydrochloric acid and phosphoric acid.

The form in which the added nitrogen is excreted depends also on whether the nitrogen is taken with the food, or injected into the body. When the ammonium salt is injected the output of urea is greatly increased, and the amount of nitrogen excreted in the faeces is much less than in the previous periods.

Professor W. H. Thompson very kindly performed the injections for us, and we are sincerely indebted to him for his valuable suggestions and advice during the course of the investigation.

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II. ON THE DEVELOPMENT OF THE BLACK MARKINGS ON THE WINGS OF *PIERIS BRASSICAE.*

By HERBERT ONSLOW.

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The formation of black animal pigments or melanins has been shown by Biedermann [1898], Gortner [1910, 1911, 1] and other investigators to be due to the oxidising action of an enzyme upon a colourless chromogen. The chromogen is probably tyrosine or a closely allied substance and the enzyme a tyrosinase.

In order to investigate the factors which determine the formation of pattern, and if possible to extend our knowledge of the mechanism of melanin production, a study was made of the black wing-markings of the common large white cabbage butterfly (*Pieris brassicae*). Hopkins [1895] has already shown that the chalky white pigment deposited in the scales of these insects gives the murexide test and is in fact uric acid. He has also shown that the yellow pigment which occurs on the under-sides of the wings of *Pieris brassicae,* and which constitutes the sole pigment of the wings of *Gonepteryx rhamni,* gives the murexide test and is a derivative—probably an oxidation product—of uric acid.

The development of the black wing-markings of *P. brassicae,* which differ slightly in the male and female, has been observed by Urech [1892], but his account is chiefly descriptive and does not deal with the cause or chemistry of their production.

Von Fürth [1904] and Roques [1909] have shown that the haemolymph of certain pupae is rich in tyrosinase, and the former has described a tyrosinase obtained from the pupae of *Deilephila elpenor* similar in most respects to that mentioned in this paper.

For the material of the present study a number of pupae of *Pieris* *brassicae* were obtained. These have a greyish green colour which remains until a short time before the emergence of the complete insect in May. A few days before this takes place the white wing-pigment makes its appearance through the chitin of the pupa-case, and a little later pale spots can be observed on the wing. If the pupa is dissected at this stage the markings do not appear black but yellowish, like spots of grease upon white paper. These markings begin to darken quickly and just before emergence become deep black. During this transition the chitinous case of the pupa becomes definitely separated from the body of the insect, so that the latter can be dimly seen lying within its semi-transparent sheath. It is probably during this period that sufficient air finds access to the wing to cause the oxidation in the black areas to take place. When fully developed this black pigment is deposited in minute granules within the chitin of the wing scales and is similar in appearance and solubility to the melanin of hair [Onslow, 1915] and other structures. It can be partially extracted by prolonged boiling in dilute alkali. It is soluble in weak alkalies, somewhat less soluble in weak acids (N/20 HCl) and insoluble in strong acids and saturated ammonium sulphate solution.

EXPERIMENTAL.

Several pupae were chloroformed just before the wing-markings began to blacken, and the haemolymph was extracted by grinding them in an agate mortar with a little kieselguhr and chloroform water. After filtration, the pale green slightly opalescent fluid obtained was made faintly alkaline to litmus with very dilute sodium carbonate solution and tubes were prepared each containing 2 cc. of the fluid. The following substances were then added to the tubes which were plugged with cotton-wool and kept at room temperature for a period of 12 hours.

Experiment I.

No.	Contents	Appearance after 12 hours
1	Haemolymph	Deep black at the surface
2	„ + tyrosine	„ „ „
3	„ + p-cresol	Blood-red
4	„ (boiled)	Unchanged

Since a deep black ring was formed at the surface of the fluid without the addition of tyrosine it is clear that the haemolymph was not only rich in tyrosinase but also in chromogen. It was found absolutely necessary to keep the tubes at a temperature of 20° or less, for at higher temperatures formation of the pigment was inhibited. This observation is in agreement with that of von Fürth [1904] who found that a temperature of 30° completely inhibited the reaction, whereas tubes kept in the ice-chest were not affected.

It is interesting to note that in vertebrates the action of the tyrosinase is accelerated by a temperature of 36°. This difference might be accounted for by assuming that the organic peroxide constituent of the oxidase of insects is completely destroyed at a temperature of 30°. Unfortunately the author has not yet been able to test this hypothesis.

In order to ascertain whether any chromogen was present in the scales, the fore-wings were dissected from a number of pupae. The members of each pair of wings are distinguished in the following experiment by the letters *A* and *B*, *B* always serving as a control. The bodies of these pupae were utilised for the preparation of the haemolymph in the manner already described. Each wing was placed in a watch-glass containing the following solutions in which they remained for 12 hours at room temperature.

Experiment II.

No. of wing		Contents of watch-glass	Appearance after 12 hours
Pair 1	A_1	Normal saline	+
	B_1 (boiled)	"	–
Pair 2	A_2	Haemolymph	+ +
	B_2 (boiled)	"	+ +
Pair 3	A_3 "	"	+ +
	B_3 "	" (boiled)	–
Pair 4	A_4	Saturated tyrosine solution	+ + +
	B_4 (boiled)	" " "	–

+ indicates slight darkening of the markings.
+ + " considerable darkening of the markings.
+ + + " blackening of the entire wing.
– " no change.

It appears from this experiment that both the wing and the haemolymph carry the two factors necessary for melanin production, since it is only when both the wing and the haemolymph are boiled that no darkening takes place (B_3). It is probable that the haemolymph which contains both chromogen and ferment adheres to the surface of the wing and may even be present in the wing nervures. When the tyrosinase of the body lymph comes into contact with the chromogen present in the wing-markings it oxidises this more than it does the rest of the wing. A fourth pair of wings was prepared, in order to determine whether the localisation which causes the formation of pattern is due to

(1) the restriction of the tyrosinase to the markings and the distribution of the chromogen over the entire wing, or

(2) the restriction to the black markings of the chromogen alone.

That it is the chromogen which is restricted to the black areas and the tyrosinase which is present over the whole surface is clear, for when the wing is placed in a tyrosine solution it becomes black all over, but when it is placed in a tyrosinase solution the darkening is restricted to the markings. This is similar to the case of the markings on the elytron of the Periodical *Cicada* described by Gortner [1911, 2]. Owing to the fact that the haemolymph contains a quantity of chromogen the contrast between the markings and the rest of the wing is somewhat obscured by the general darkening. On microscopical examination however the pigment of the markings can be seen within the chitin of the scales, whereas the pigment deposited on the white portions of the wing lies in irregular masses on the surface of the scales. Fortunately however it was possible to make the reaction far more marked by precipitating the tyrosinase from the haemolymph with ammonium sulphate solution and by very thoroughly washing the precipitate with the same fluid so as to remove the last traces of chromogen. If the precipitate is now dried between filter papers and re-dissolved in an equivalent amount of 0·05 % sodium carbonate solution, a fluid containing tyrosinase is obtained free from chromogen.

An attempt was made to substitute the tyrosinase prepared from the skins of young black rabbits [Onslow, 1915] but the result was not successful, owing no doubt to the difference in the tyrosinase of warm-blooded animals.

SUMMARY.

The black markings on the wings of *Pieris brassicae* are caused by the oxidation of a colourless chromogen by a tyrosinase. This ferment is supplied from the body-lymph of the pupa, possibly by means of the wing-nervures, to the chromogen which has previously been deposited in the areas destined to become black. The form of the markings is determined by the localisation of the chromogen to these areas. The oxidation takes place just before the emergence of the fully-developed insect and as soon as the atmospheric oxygen has access to the surface of the wing.

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III. THE DISTRIBUTION OF MALTASE IN PLANTS. I. THE FUNCTION OF MALTASE IN STARCH DEGRADATION AND ITS INFLUENCE ON THE AMYLOCLASTIC ACTIVITY OF PLANT MATERIALS.

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In an investigation of the sugars present in the leaves and stems of various plants [Davis, Daish and Sawyer, 1916; Davis and Sawyer, 1916] it was found that maltose was invariably absent. Many different types of plants were examined, some of which, for example the potato, turnip, *Tropæolum* and sunflower, elaborate large quantities of starch in the leaf during the day-time to be used as a reserve during the night. If the degradation of the starch were effected merely by the ordinary diastatic enzymes, which convert starch into maltose and dextrin, maltose should be found in the leaves or circulating system of these plants, especially at night. In no single case, however, throughout the night or day could even traces of maltose be detected in starch-forming plants. Negative results were also obtained with other plants such as the mangold, dahlia and grape vine which store reserve carbohydrates other than starch (saccharose, inulin and glucose). For the first time in work of this kind a method of analysis has been used, based on the action of selective yeasts, such as *S. marxianus* and *S. exiguus* [Davis and Daish, 1913], which enables maltose to be estimated accurately in presence of other sugars such as saccharose, glucose, laevulose and pentoses. From the results of some 500 analyses, we can affirm with certainty that maltose was entirely absent from the leaf and stems of all the plants we examined. The writer is of opinion, on grounds which will shortly be stated, that this absence of maltose will be found to be a general if not universal phenomenon in plants.

From the work referred to above the writer has been led to extend the views put forward by Brown and Morris [1893] as to the mechanism of starch degradation in plants, by assuming that the enzyme *maltase* plays a part also in the transformation of starch into soluble sugars. This enzyme, instead of having a very limited distribution as assumed hitherto, probably takes part invariably in breaking down the starch in plant tissues. This degradation undoubtedly takes place in a series of successive stages, of which at least three may be distinguished:

(1) Starch ⟶ soluble starch and dextrins ("*Liquefying*" *enzymes*).
(2) Dextrins ⟶ maltose (*Dextrinase*).
(3) Maltose ⟶ glucose (*Maltase*).

The Presence of Maltase in Foliage Leaves and Seeds.

The fact that maltose was invariably absent from the leaves and circulating vessels of plants suggested that experiments should be made to ascertain whether maltase existed in foliage leaves, especially during the times when starch degradation is at its maximum. Such experiments carried out in this laboratory by A. J. Daish have shown that this is invariably the case; they are described in the following communication [Daish, 1916, 1].

The part played by maltase in the germination of seeds in which large stores of starch are gradually utilised has been hitherto almost entirely ignored. Since the pioneer work of Brown and Morris [1890] on the germination of some of the Gramineæ it has been always assumed that the starch is resolved by the enzymes of the seed simply into maltose and dextrin, and all calculations as to the development and activity of the enzymes in germinating seeds have been based on the assumption that a *single* reducing sugar, maltose, is formed. In the very careful work of Brown and Escombe [1898] and more recently of Stoward [1911] on the distribution of enzymes in the barley grain during germination, the fact that two saccharifying enzymes are present, capable of forming *two* sugars, has been left out of consideration. In the writer's opinion, if this fact had been recognised, certain results obtained by Stoward which are at present difficult to understand, such as the power possessed by the non-living inner endosperm of augmenting its amylolytic activity, would have found an explanation.

The results obtained by A. J. Daish [1916, 2] clearly show that in germinating barley considerable quantities of maltase are present and that the starch of the grain is resolved by this enzyme finally into glucose. We are conscious that these experiments could have been made more complete in several directions, but as they were carried out more than two years ago, being interrupted by the war, and there is little prospect that further work can be done in the immediate future, they are published as they stand.

Maltase was first discovered in maize by Cuisinier [1885, 1886] who termed it "glucase" and patented a process of converting starch into glucose based on its use. The later work of Geduld [1891], Beyerinck [1895], Morris [1896], Huerre [1909] and Wierzchowski [1913, 1, 2] has confirmed that of Cuisinier and shown that maize is particularly rich in maltase. Regarding the presence of maltase in other cereals, especially barley, there has been a great divergence of opinion which it is now necessary to explain.

The experiments of Butkewitsch [1908] on the enzymes present in the barks and woods of plants during the spring period of starch dissolution are interesting as showing the probable presence of maltase in these parts, although Butkewitsch does not specifically recognise this. Such bark, *or even an aqueous extract of it*, hydrolyses starch and maltose; but when the extract is precipitated by alcohol the property of acting on maltose is destroyed. It is clear that this is due to the presence of *maltase*. The influence of chloroform and toluene in promoting starch dissolution is shown; when twigs are cut and stored in water containing toluene or chloroform, the starch dissolving enzymes rapidly come into action and remove the starch stored in the rind. In water alone the starch is not affected. Toluene has a far greater effect than chloroform, no doubt owing to the fact that the latter largely destroys the maltase. Butkewitsch's experiments [1908, p. 339] showing the limitation of the starch dissolving action by the presence of glucose are also important.

Failure of earlier Workers to detect Maltase in Cereals.

The failure of earlier workers to recognise the universal distribution of maltase in cereals is largely due to the fact that it is an endo-cellular enzyme which does not readily diffuse out of the plant cells and is not easily extracted by water. Extracts of the cereals (with the exception of maize) as a rule have little action on maltose and produce only small quantities, if any, of glucose, from maltose or starch. Many workers have failed to detect maltase owing to its instability. They have worked under conditions which destroyed the enzyme. Thus they used enzyme preparations which had been precipitated by alcohol or worked in presence of chloroform as an antiseptic, both of these substances *under ordinary conditions* rapidly destroying maltase.

It seems probable that *pure* alcohol and *pure* chloroform would not destroy maltase and that their usually destructive action is due to the presence of small quantities of acid or alkali. Ordinary chloroform, when kept in a white glass bottle, frequently contains large quantities of hydrogen chloride and carbonyl chloride; when its decomposition into these substances is prevented by adding lime, the chloroform may become distinctly alkaline. Ordinary alcohol is generally acid, but in some cases, when stored in soft glass bottles, it becomes alkaline. Differences in the nature of the chloroform would explain the fact that Tebb [1894] succeeded in extracting maltase from animal tissues with chloroform water whereas Brown and Heron [1880] failed; in the latter case it is probable that the chloroform used destroyed the enzyme. In the same way the contradiction between Fischer's [1894] positive results as to the action of yeast on maltose in presence of chloroform and his later negative results [1895] under the same conditions, which confirmed the equally negative results of Morris [1895], may be explained. Similarly Beyerinck [1895] succeeded in precipitating the maltase of maize in an active condition with alcohol and Marino and Sericano [1905] the maltase of germinated barley; whilst Huerre [1909] and Wierzchowski [1913, 1, 2] state that this method gives with maize a preparation which contains little active maltase but behaves like ordinary diastase, converting starch into maltose, a result clearly due to destruction of the maltase. Our own experience [Daish, 1916, 2, p. 65] with germinated barley has been similar to that of Wierzchowski with maize.

In other cases the action on starch has been studied at a temperature of 55–60°, at which the maltase is either destroyed or at least has its activity greatly restricted. Huerre [1909] has shown that the maltase of the "*blanc hâtif*" maize of the Landes is very rapidly destroyed at 65° and somewhat less rapidly at 50° (completely in 2 hours). In other varieties of maize the maltase was found to be more resistant, probably owing to the presence of protective substances. The writer [1914] has shown that the very active maltase of taka-diastase has its power of converting maltose enormously restricted at 55°. Beyerinck's [1895] maltase from maize resisted a temperature of 55°, whilst the maltase prepared from yeast was rapidly killed at this temperature.

As a rule, the optimum temperature of plant maltases lies in the neighbourhood of 38–40° as in the case of yeast maltase; above 50° rapid weakening occurs. In some cases, however, for reasons which have not been ascertained, certain plant maltases, for example the "*haute maltase*" of maize "*jaune hâtif*" [Huerre, 1909], show a higher temperature of optimum action.

Bearing the above facts in mind it is possible to explain all the contradictory statements as to the occurrence of maltase in leaves or in germinating or resting seeds. When maltase has *not* been found it has been due to the method of working either failing to extract the enzyme or destroying it. It is interesting historically to note that Brasse [1885] prior to the work of Cuisinier and Geduld on "glucase" clearly realised that the "diastase" isolated from germinated barley or from foliage leaves is capable of digesting *raw* starch to glucose, if the digestion *takes place at a low temperature* (34–42°). He points out that, providing the *extract is made rapidly in the cold* and "*subisse le moins longtemps possible l'action de l'alcool*" used to precipitate it, a product is obtained which effects the complete degradation of starch to glucose. Brasse, however, did not realise that a special enzyme (maltase) is concerned in the change as did Cuisinier shortly after, nor did he show that it acts by breaking down the maltose formed from starch by other enzymes. He realised, however, more clearly than later workers, the relative instability of the active enzyme and, in particular, points out that no glucose is formed at 50–57°. Had Brasse's paper been properly appreciated many of the contradictory statements of later workers would have been avoided.

It is not necessary here to analyse in detail the work of all those authors who failed to find maltase in plant material. Brasse, Cuisinier and Geduld considered that *all* cereals contain a glucose-forming enzyme (*glucase*). Lintner and Eckhardt [1889] contrasted the properties of the diastase of ungerminated barley with those of the diastase of malt, and although they concluded that the former gave rise only to maltose and dextrin, it would appear from the fact that they found the optimum temperature of activity (45°) to be considerably lower than that of malt diastase and a great falling off of saccharifying power to occur above 50°, that they worked with a preparation containing maltase. Later Lintner [1893] confirmed Cuisinier's statements [1885, 1886] as to the presence of "glucase" in maize and stated that glucose may also be formed in not inconsiderable quantities by the action of dried malt on starch. Brown and Morris [1893] in the case of foliage leaves observed only a transformation of the starch into maltose, and Morris [1896] stated that he had examined a considerable number of malted and unmalted grains but could find no evidence of a maltase. Beyerinck [1895] denied the general distribution of maltase in leaves and seeds (e.g. barley, wheat and rye) but found it to be present in the grain of maize, sorghum and millet. There is no doubt that in these last seeds it is especially abundant so that it could hardly escape detection even under adverse conditions of working. Later workers like Brown and Escombe [1898], Ling and Baker [1895, 1897 and

1902], B. F. Davis and Ling [1904], Ford [1904], Ford and Guthrie [1908] have all ignored the possible presence of maltase in barley and malt. On examining the conditions under which these authors worked it is clear that their negative results are to be attributed to their not realising the instability of the enzyme and the difficulty of extracting it.

Beyerinck [1895, p. 338] seems to have understood the difficulty of extracting "glucase" with water. Thus he says that rice meal contains much glucase, but on attempting to extract it he obtained, much to his surprise, an enzyme which acted strongly on starch but converted it only into maltose, without forming glucose. A similar result was obtained with oats. He is inclined to believe "though proof of this is difficult" that in the process of purification adopted, "glucase" is converted into "*granulase*" (that is, the enzyme which produces maltose and achroodextrin from starch). Obviously the maltase had been destroyed leaving behind the less sensitive ordinary diastatic enzymes.

The recent work of Wierzchowski [1913, 1, 2], published since our own was undertaken, clearly proves that all cereals contain considerable quantities of maltase, although as a rule it cannot be extracted with water owing to its endo-cellular nature. In buckwheat, millet and maize it is particularly abundant; in rye, barley, wheat and oats less so.

Wierzchowski realising the insolubility and instability of the enzyme made his experiments in the same way as we have done: the *finely-ground meal* was allowed to act upon the starch or maltose solution for 24 hours at 45° in presence of formaldehyde as antiseptic. The glucose formed was estimated as osazone; as the total reducing power was also determined, the course of action could be followed. The polarimeter was not used.

As will be seen later, Wierzchowski incorrectly interprets his results, in so far as he considers that his "maltase" could act directly upon starch, as well as upon maltose; the whole of his results, as well as our own, are best explained by the view that the maltase simply breaks down maltose which has been formed from starch by the other diastatic enzymes (see p. 39).

Presence of Maltase in Malt and Malt Extracts.

Germinated barley as Daish's [1916, 2] results show, as well as those of Brasse [1885], Marino and Sericano [1905], Marino and Fiorentino [1906] and Wierzchowski [1913, 1, 2], undoubtedly contains maltase. Whether a malt or malt extract made from such barley contains maltase or not will depend upon circumstances. If the malt be dried at a low temperature, the maltase will largely survive, but if it be kilned at a high temperature, part, if not the whole, of the maltase may be destroyed, so that such malt will contain principally the ordinary diastatic enzymes which break down starch only to the stage of maltose. On the other hand, kiln drying, if not too prolonged or carried out at too high a temperature may actually facilitate the extraction of maltase *by water*, by rupturing the cells or destroying the protoplasm in which the enzyme is imprisoned, just as drying facilitates the extraction of maltase from yeast, which fails to yield its enzyme when in a fresh condition. These views would explain the idea of Ling and Baker [1895] that kilning confers upon malt the power of converting maltose into glucose and that air-dried malt does not possess this property; Kröber [1895] asserted exactly the opposite. It is possible to reconcile these two statements by assuming maltase to be present which was rendered more easily soluble under Ling and Baker's conditions of malting and destroyed under those of Kröber. It is equally possible to understand Morris' statement [1896] that Ling had later been unable to confirm the observations of Ling and Baker that "the ability to convert maltose into dextrose which some malts possess is conferred by the kilning process and we may therefore take it that a normal malt contains no enzyme capable of hydrolysing maltose."

Most malt *extracts* probably contain little or no maltase, owing both to the difficulty of extracting this enzyme by water and the fact that it is readily destroyed during either kilning or extraction and concentration, especially if evaporation is carried out at too high a temperature (above 45°). Brasse [1885] stated that although germinated barley contained a glucose-forming enzyme, this was entirely absent from all the commercial malt extracts he had examined.

Whilst maltase is probably absent from most commercial malt extracts this should not be presumed in the case of any sample without proof. ***Fine grinding*** of malt, by rupturing the cells which contain the maltase, may increase the amount of the latter which is extracted by water. Thus Wierzchowski [1913, 2] by extracting maize which was ground to different degrees of fineness obtained the following comparative results for the glucose formed from maltose:

Coarse grinding (above 2 mm.)	6·0 % glucose
Less coarse (above 0·5 mm. sieve) ...	28·0 ,,
Fine (below 0·5 mm. sieve)	37·5 ,,

It is well known that the diastatic activity of a malt differs considerably with the fineness of grinding, a fact which is probably due in part to differences in the amount of maltase passing into solution.

Formation of Glucose by the action of Malt or Barley Diastases on Starch.

Ling and Baker's [1895] statement, that the diastase of high dried malt produces glucose whereas low dried malt does not, has been explained above. As a matter of fact low dried malt contains a considerable amount of maltase but it is not readily extracted. Baker [1902] working at 50° with the precipitated diastase of ungerminated barley found glucose to be formed from soluble starch after more than 6 hours; as the precipitated diastase failed to act upon maltose but converted "a-amylodextrin" into maltose and glucose, he considered that the glucose was formed from the dextrin and not from maltose. It is probable that Baker's enzyme, although it had been precipitated by alcohol and so weakened, contained some maltase, which in the long periods of digestion in his experiments gave rise to glucose. The action would in fact be similar to that of taka-diastase, which contains maltase, at 55° [Davis, 1914]; although the activity of maltase is greatly impaired at this temperature it is still capable of forming 20 % of glucose from starch after 50 hours (1 % solution of starch, 0·1 g. taka-diastase). The fact that maltose was not attacked by Baker's weakened enzyme is not surprising and may be attributed to impurities in the maltose used[1].

B. F. Davis and Ling [1904], who found glucose to be formed from starch by the action of "restricted" malt diastase, worked with a precipitated diastase prepared from a malt dried below 33°, which therefore contained undestroyed maltase; they considered that when this diastase had its activity "restricted" by heating for a short time at 60–70°, the enzyme was so modified in its nature as to give glucose among the products of its action on starch. It is more probable that the glucose owed its origin, in the prolonged periods of digestion adopted, to traces of maltase which escaped destruction during the "restriction." The slow formation of glucose under these conditions is quite parallel to the results obtained by partly destroying the activity of taka-diastase by heating at 55°. That the heating operation, which was supposed to confer the power of forming glucose, actually destroys maltase appears from the gradual falling off of the power of forming glucose with the time of heating. "Restriction" at 70·5° for example gave an enzyme which after 67 hours' action on starch gave the following quantities of glucose (measured as osazone): 5 minutes' "restriction," 10·8 %; 17 minutes', 10·0 %; 30 minutes', 7·3 %; 120 minutes', 3·9 % [B. F. Davis and Ling, 1904, Series vi, vii and viii].

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[1] Maltose recrystallised several times from alcohol in *ordinary* glass may take up sufficient alkali to destroy the very sensitive maltase. The writer has had preparations of maltose which failed to yield glucose with maltase on this account; maltose to be used in such experiments should be recrystallised in Jena glass vessels.

Ling [1907, p. 182] considers that the action here discussed was not due to maltase, as the heated diastase solution did not convert maltose into glucose; but this was probably owing to the reason given above. In their recent work on the action of precipitated diastase from kiln-dried malt on starch granules Baker and Hulton [1914] generally observed the formation of glucose at low temperatures after digestions of 4 days or more. There is little doubt that this was due to the presence of traces of maltase which escaped destruction during the precipitation of the diastase by alcohol, although they regard the glucose as formed from dextrins of low molecular weight. They consider that "it is unlikely that maltose can undergo hydrolysis by an amylase separated from malt."

Ling and Rendle [1904] stated that commercial concentrated malt extracts invariably contain glucose. Part of this glucose is probably formed from the starch of the malt by the action of maltase, but as was pointed out by Beyerinck [1895] it is probable that part arises from the inversion of saccharose, which is invariably formed in barley during germination. After extraction, the saccharose would undergo inversion by the invertase present in the extract.

The Maltase of Plants does not act directly on Starch or Dextrin but only on Maltose.

Most of the investigators who have discussed the formation of glucose from starch have considered that this sugar may be split off from the starch or dextrin molecule by the direct action of an enzyme, without the prior formation of maltose. Thus Beyerinck [1895] held that the "glucase" of maize might act directly on starch forming dextrin and glucose; also that it is capable of resolving dextrin into maltose and glucose. Ling and Baker in their various papers expressed a similar view that glucose might be formed directly from amylodextrin [compare Baker and Hulton, 1914]. Kita [1913] held a similar view with regard to the action of taka-diastase or "koji" on starch. The writer [1914] has shown that in this case glucose is undoubtedly produced from pre-formed maltose; as the glucose appears an equivalent quantity of maltose disappears and the action is perfectly continuous.

In the case of the enzymes of germinated barley it is not so easy to give a definite proof that the glucose arises only from maltose, as the dextrin stage persists longer than with taka-diastase; as shown by Daish [1916, 2; Table VI, p. 67], dextrin is present even in the later stages (263rd hour) of the action. The fact that the starch of the barley itself undergoes slight but progressive digestion is an additional complication, it being impossible to correct for the sugars formed from the starch in a rigidly exact way by a "control" experiment as the self-digestion of the barley is greatly modified by the presence of maltose or starch [Daish, 1916, 2, p. 70]. The following considerations founded on the data in Tables VI and IX of Daish's paper [1916, 2, pp. 67, 71] make it highly probable, however, that the glucose is derived entirely from maltose and not from starch or dextrin.

TABLE I.

Action of 0·2 *g. Germinated Barley on Maltose—Increments of Glucose compared with Maltose Disappeared.*

Interval	Time, hours	Glucose formed, g.	Maltose calc. as equivalent to glucose formed, g.	Decrease of maltose, g.	Remarks
1	0–119	0·4268	0·4054	0·4170	Maltose corr. with glucose nearly = maltose transformed
2	119–191	0·1497	0·1422	0·1347	Maltose corr. with glucose > maltose converted
3	191–263	0·0945	0·0898	0·0615	Maltose corr. with glucose much > maltose converted

TABLE II.

Action of 0·2 *g. Germinated Barley on Starch—Increments of Glucose.*

Interval	Time, hours	Glucose formed, g.	Maltose corr. with glucose, g.	Maltose apparently transformed, g.	Remarks
2	119–191	0·1019	0·0968	0·0645	Maltose corr. with glucose > maltose apparently converted
3	191–263	0·0885	0·0840	0·0273	Maltose corr. with glucose much > maltose apparently converted

The tables given above show the increases of glucose (calculated as their equivalent in maltose) compared with the maltose which has disappeared in the successive intervals during the transformation of maltose and starch by 0·2 g. of germinated barley [see Daish, 1916, 2, Sections vi and vii].

As stated on p. 32 we regard the degradation of starch as taking place in a series of successive stages, each characterised by an appropriate enzyme:

Starch ⟶ soluble starch ⟶ dextrins ⟶ maltose ⟶ glucose.

All these changes run concurrently during the whole time of action, but the relative velocities of the different changes are considerably modified by alterations in the concentration of the products of action at different periods. The fact that when germinated barley is used the starch of the latter is itself hydrolysed, especially during the later stages of action, is an additional complication. In the relatively simple case of the hydrolysis of pure maltose, this factor has no great disturbing effect in the early stages of the change, as the excess of maltose largely inhibits the self-digestion of the starch [see Daish, 1916, 2, Section vii]; consequently the glucose formed corresponds with the maltose that has disappeared (actually in the first 119 hours, see Table I, the glucose formed, calculated as maltose, 0·4054 g. is slightly less than the maltose which has disappeared, 0·4170 g.). In the later stages, however, with both maltose and starch, the glucose formed, calculated as maltose, is considerably greater than the maltose apparently transformed. This is no doubt due to the fact that in these periods, as the original maltose concentration falls, more and more of the starch of the barley undergoes transformation into maltose, so that there is a new formation of maltose to replace that transformed into glucose.

That the glucose is formed entirely from pre-formed maltose even in the case of the starch hydrolysis appears clearly on plotting the data in Tables I and II, so as to show the rate of formation of glucose from starch as compared with that obtained in the case of pure maltose (Fig. 1, p. 42). With starch this curve is practically a straight line from the 119th to the 263rd hour. With maltose, the curve can be plotted from the beginning of the change and it is seen that up to about the 180th hour the rate of formation of glucose gradually falls, but subsequently the glucose is formed along a straight line which almost coincides in position and direction with that showing the formation of glucose from starch.

In no period of the starch transformation is the rate of increase of the glucose greater than that found in the maltose transformation, so that there is no evidence pointing to a direct formation of glucose from starch. In fact during the first stage, up to the 180th hour, the rate of increase of the glucose is greater in the case of the maltose hydrolysis than in that of starch as shown by the greater steepness of the curve. This is well marked from the 119th to 180th hour when the *concentration* of the glucose present is less in the case of the maltose curve than in that of starch; it is this concentration which determines the rate of formation of fresh glucose. During the period from the 180th to 263rd hour, *when the concentration of the glucose is the same in both cases,* the curves for starch and maltose are practically coincident and form strictly parallel straight lines.

Fig. 2 shows the rate of *apparent* disappearance of maltose in the two hydrolyses. With starch the curve is less steep than with pure maltose. This is no doubt due to the fact that, in the case of starch, maltose is being formed from dextrin at the same time as it is being transformed into glucose by the maltase of the barley. Maltose therefore *apparently* disappears less rapidly in this case than in that of the maltose alone.

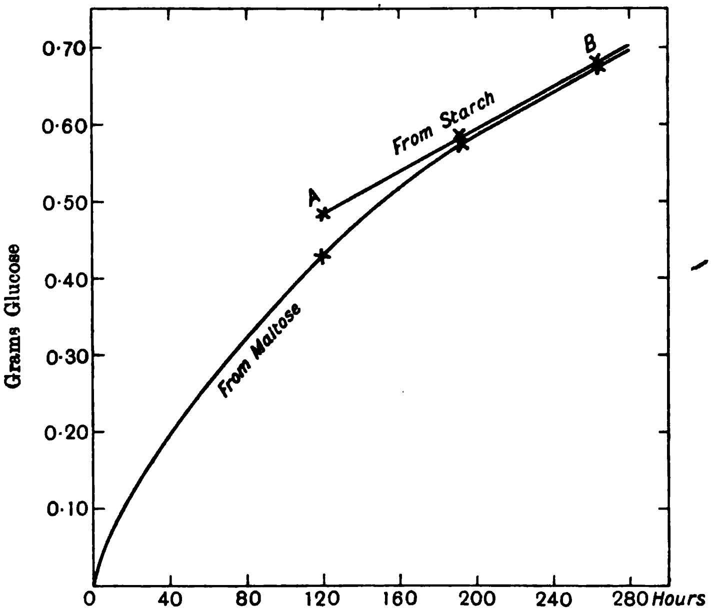

Fig. 1. Formation of Glucose by action of Germinated Barley (0·2 g.) on Maltose and Starch at 38°.

There is therefore no reason to believe that the glucose formed in the transformation of starch ever arises directly from the starch *or in any other way than by the action of maltase on pre-formed maltose.* The action of the germinated barley in this respect is exactly similar to that of taka-diastase or "koji." Beyerinck's [1895] results which were supposed to prove the contrary can be explained by the view that his precipitated "glucase," *which attacked starch and dextrin,* contained sufficient of the ordinary amyloclastic enzymes to produce the maltose on which the "glucase" could act. That this was the case appears clearly from his statement [Beyerinck, 1895, p. 335] that the formation of glucose from soluble starch or dextrins is less rapid than from maltose. In his purified "glucase" the proportion of the ordinary amyloclastic enzymes which form maltose was relatively less than that of maltase, so that the rate of formation of glucose in the case of the starch was limited by the small rate of production of maltose. Maize indeed seems to contain the liquefying and maltose-forming enzymes in relatively small amount as compared with other cereals[1] such as barley and the maltase in relative excess, although, as the results of Wierzchowski [1913, 1] show, *finely ground maize meal* which has *not been first extracted with water* to remove these enzymes contains sufficient of the ordinary diastases to form maltose faster than it can be dealt with by the maltase.

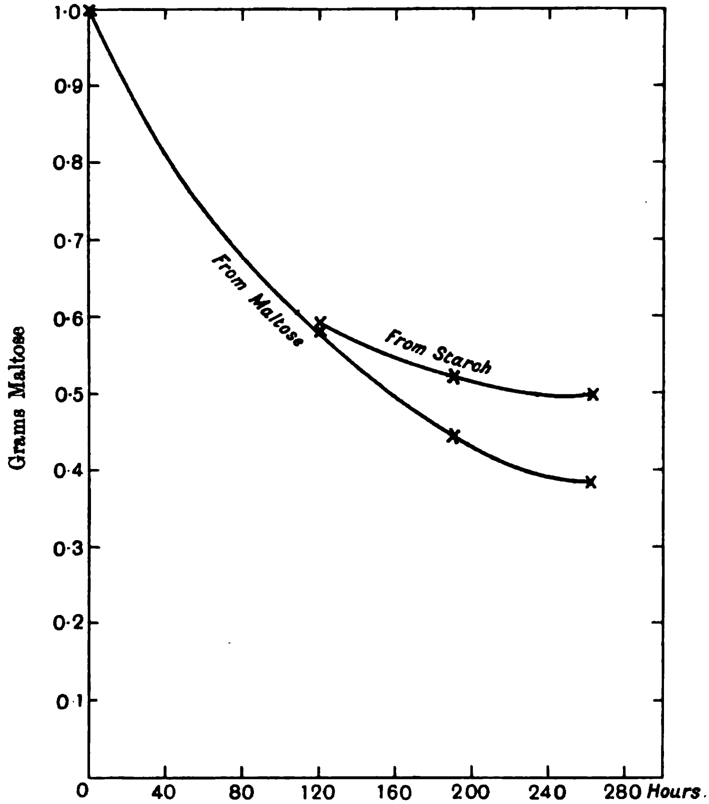

Fig. 2. **Fall of Maltose by action of Germinated Barley on Starch and Maltose at 38°.**

Wierzchowski [1913, 1] commenced his work on the action of maize-maltase on starch in the hope of being able to split off glucose directly from the latter by rupturing the γ-bonds of Syniewski's formula for starch. He found instead that action occurred, in two stages; in the first maltose was formed and in the second glucose. He correctly attributed this to the presence of the ordinary diastatic enzymes producing dextrins and maltose. In the first stage of the action, maltose is formed more rapidly by these enzymes than it can be converted by the maltase, so that it accumulates[1]. He then extracted the ordinary "diastases" from the meal, completely as he thought, by water and subjected the starch to the action of the extracted meal. He now obtained an entirely different result, glucose apparently being produced without maltose, and considered that he was effecting a pure γ-hydrolysis. The fallacy underlying this view is that in all probability he had failed *completely* to remove the ordinary maltose-forming enzymes by his preliminary extraction with water. What really happened was that these enzymes were left in relatively small proportion to the insoluble maltase present, so that when the *extracted* meal acted on starch, maltose was formed from starch far less rapidly and, *as fast as it was formed,* could be dealt with by the excess of maltase present and transformed into glucose. Consequently maltose did not accumulate. An examination of Wierzchowski's data (glucosazone yields) shows that, even in those experiments in which glucose was thought to be formed directly, some maltose was formed, so that it is clear that he had not entirely removed the ordinary amyloclastic diastases.

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[1] This appears also from Cuisinier's patent [1885]. In order to facilitate the formation of glucose from maize, the first stages of the action are hastened by the addition of *malt*, which supplies the ordinary diastatic enzymes.

In the writer's opinion, there is no reason at present to assume that glucose is ever split off directly from starch or dextrins or that the degradation of starch takes place otherwise than by the series of changes:

Starch ⟶ soluble starch ⟶ dextrins ⟶ maltose ⟶ glucose,

each of which changes corresponds with the activity of a special enzyme. The localisation of the different enzymes in the seed is in accord with this view and may be briefly discussed.

Localisation of Maltase in Plant Seeds.

Brown and Morris [1890] at first regarded the scutellum of the embryo as the principal seat of diastatic activity and considered that the aleurone layer was not actively secretive; in later work Brown and Escombe [1898], however, concluded that the aleurone layer has real secretive functions, a view supported by the recent work of Stoward [1911] which tends to show that the aleurone layer has actually a far higher amyloclastic activity than the embryo. In all this work, however, the enzymic activity has been measured on the assumption that only a single sugar—maltose—is formed during the starch transformation; the activity has been expressed in terms of reducing power only, without reference to the actual products of the action. It is unfortunate that Stoward in his recent very detailed work on the barley grain did not realise that in the depletion of the grain the enzyme maltase plays an important part, giving rise to glucose, a sugar with nearly twice the reducing power of maltose. Stoward's digestions to measure the amyloclastic power were carried out at 30°, a temperature at which the maltase could act.

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[1] The action of the maize meal is in these respects exactly similar to that of taka-diastase [Davis, 1914]; in both cases the breaking down of starch to maltose gives rise at first to far more maltose than can be immediately transformed. In the case of taka-diastase, dextrin disappears after the first 3 or 4 hours, and after this period the change consists solely in a transformation by maltase of maltose into glucose. The behaviour of taka-diastase towards starch under different conditions is the key to the whole question of the nature of the plant enzymes which effect starch degradation.

Neither Stoward nor Brown and Escombe have referred to the early but very important work of Beyerinck [1895] on maize, which although giving, as we have shown above, incorrect views as to the nature of starch degradation and the general distribution of maltase, clearly indicates a different place of origin of the different enzymes in the grain. Beyerinck's work is extremely interesting as suggesting that the aleurone layer and embryo secrete in the main *different* enzymes[1]. The scutellum secretes mainly *granulase*, that is the starch-splitting enzyme, especially at the commencement of germination. The aleurone layer is the source of the "glucase," that is the maltose-splitting enzyme. Exactly the same differentiation of function is found in sorghum. According to Wierzchowski [1913, 1] also the "maltase" is mainly localised in the aleurone layer of maize, from which it is best prepared. The embryo, although active in producing the ordinary diastatic enzymes, contains little or no "glucase" (maltase); this enzyme is mainly elaborated by the aleurone cells. It is impossible in the present state of our knowledge to criticise these views, but it is highly probable that different cells secrete the ordinary diastases and the maltase of seeds during germination. It is certain that in future work on the localisation of the amyloclastic enzymes in seeds, the part played by maltase must be more fully recognised than it has been hitherto.

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[1] In Beyerinck's nomenclature *granulase* is the enzyme which transforms starch into maltose and *achroodextrin*; *maltase* is the enzyme which forms maltose and *erythrodextrin*; *glucase* forms glucose (either from starch, dextrins or maltose). *Dextrinase* converts granulose into "malto-dextrin" only. "Amylase" is a collective name for all the starch-splitting enzymes. Beyerinck in general names his enzymes according to the product they form: his *maltase* forms maltose, and must be distinguished from the enzyme now generally called maltase, which is capable of resolving maltose (and α-glucosides) *only* into glucose. This function is given by Beyerinck to "glucase," but as shown above this author is in error in regarding this enzyme as capable of direct action on starch and dextrin. Wierzchowski is equally in error in assuming that his "maltase" (which corresponds more closely to the modern maltase) acts directly on starch or dextrin (see p. 44).

Measurement of Amyloclastic Power (Diastatic Power) of Cereals, Malts and Diastase Preparations.

In order to obtain accurate comparative values of the diastatic activity of plant tissues or enzymic preparations it is necessary to take into account the possible presence of maltase. When maltase is present (air-dried barley, germinated or otherwise, green malts, taka-diastase, pancreas preparations) which gives rise to glucose, a sugar with nearly twice the reducing power of maltose, it is not sufficient to measure the cupric reducing power only after the transformation, but it is necessary to take into account the actual products of the change. Thus in the case of taka-diastase, which contains much active maltase, the measurement of the cupric reducing power only without reference to the specific rotatory power has led to entirely erroneous ideas as to the nature of this enzyme (e.g. by Stone and Wright [1898]). It is true that probably most of the commercial diastase preparations (malt extracts, precipitated diastases) contain very little maltase, owing to its destruction or non-extraction during the process of manufacture; but the recent work of Sherman and Schlesinger [1912] shows that pancreatic preparations, even after the processes of purification and concentration adopted, may contain a considerable amount of maltase which has escaped destruction.

With plant material such as ground barley or malt, the ordinary method of measuring diastatic activity by means of an aqueous extract probably gives a very incorrect idea of the total diastatic enzymes: this is clear from the work of Ford and Guthrie [1908] who showed that by using papain digestion in the preparation of the extracts far higher results (varying from two to sevenfold) were obtained in the case of barley. The ordinary aqueous extraction measures only the *easily soluble diastatic enzymes* which form maltose; papain extraction not only renders soluble the endo-cellular enzymes such as maltase but preserves them in solution. This preservative action is one of the principal functions of the papain in such cases; as was shown by Ford and Guthrie, high values are also obtained by using boiled papain solutions of which the digestive activity had been destroyed. How much of the increased activity in Ford and Guthrie's experiments was due to maltase is not clear: they worked at a temperature of 30°, at which maltase would be active, but they distinctly state that glucose was not formed in their experiments.

The Lintner method of determining diastatic activity at 70° F. (21° C.) would allow maltase to act, but not under optimum conditions. In Sherman, Kendall and Clarke's [1910] method on the other hand the temperature prescribed, viz. 40°, is that of the optimum activity of maltase. In the case of diastatic agents rich in maltase, such as taka-diastase, the two methods would probably give very different results. Tests carried out at 55° at which the activity of maltase is largely destroyed would again give rise to a totally different measure of diastatic activity.

Fine grinding in the case of barley or malt, as shown on page 37, would increase the diastatic activity by liberating or rendering soluble a larger proportion of endo-cellular enzyme.

Self-digestion of Barley or Malt.

It is clear from Daish's results [1916, 2, Sections i and v] that the self-digestion of germinated barley or malt leads finally to the production of large quantities of glucose. In the early stages dextrins and maltose are also formed, but as time proceeds these give place more and more to glucose, as the action of maltase continues.

SUMMARY.

1. Maltase is probably present universally in plants in which starch degradation occurs. The failure of earlier workers to detect it is due to the fact that this enzyme is endo-cellular and therefore not easily extracted by water; and to its instability. It is easily destroyed by ordinary alcohol or chloroform. Its action, too, is greatly limited or even destroyed at temperatures above 50°.

2. Maltase occurs in considerable quantities in germinated and ungerminated cereals. In malt it may sometimes occur, if the kilning has been at a sufficiently low temperature not to destroy it. In such cases the kilning may actually render the maltase more easily extractable by water. The presence of maltase in malt or malt diastases would explain the formation of glucose from starch which has previously been attributed to other causes.

3. It is shown that the maltase of plants does not act directly on starch or dextrins but only on the maltose, which has been formed by the ordinary diastatic enzymes; no direct splitting off of glucose from starch ever occurs. When such appears to be the case, as in Beyerinck's and Wierzchowski's experiments, maltose has been formed by the previous action of the ordinary diastases and has been then acted on by maltase.

4. The action of the enzymes of germinated barley on starch is very similar to that of taka-diastase, only taka-diastase is richer than the barley in maltose-forming enzymes so that the dextrin stage is passed through more rapidly. In both cases the glucose is formed by the action of a maltase on maltose.

5. It is probable, from Beyerinck's results with maize, that the maltase is localised mainly in the aleurone layer of the endosperm.

6. In determining the diastatic activity of plant material and preparations such as taka-diastase and pancreatins, the presence of maltase should be taken into account.

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IV. THE DISTRIBUTION OF MALTASE IN PLANTS. II. THE PRESENCE OF MALTASE IN FOLIAGE LEAVES.

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In Brown and Morris' [1893] classical paper on the "Chemistry and Physiology of Foliage Leaves" the early history is traced of the question of the starch resolving enzymes present in plants. Brown and Morris proved in a conclusive manner that the starch in the leaf is dissolved by the action of a diastatic enzyme; they regarded the formation of this enzyme as a "starvation phenomenon." They showed that diastase was present in a large number of plants and concluded from a study of the sugars of the leaf, amongst which maltose was always found, that the degradation of starch is effected in the same way as by malt diastase, the end product being maltose.

As explained by Davis [1916, p. 31] the fact that maltose was never found among the sugars present in a large number of leaves examined at Rothamsted [Davis and Sawyer, 1916] led to a re-examination of the enzymes of the leaf. This has shown that in addition to the ordinary diastatic enzymes, which convert starch into dextrin and maltose, the enzyme *maltase* is also invariably present. The absence of maltose from plant leaves at all periods of the night and day is therefore easily explained. It is due to the fact that sufficient maltase is always present to convert the maltose, as fast as it is formed from starch, into the final product of hydrolysis, glucose.

It is interesting historically that Brasse [1884] in a paper cited by Brown and Morris [1893] showed that the diastatic action of leaves on starch paste at 63° gives rise to maltose[1] and dextrin just as does that of malt diastase.

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[1] At 63°, the maltase was destroyed, leaving the ordinary diastatic enzymes: hence the production of maltose and dextrin [see Davis, 1916, p. 34]. At 34–42° the maltase remained and converted the maltose into glucose.

In a subsequent paper [Brasse, 1885] which Brown and Morris apparently overlooked, it is, however, shown that a "diastase" can be extracted from leaves and germinated barley *which at* 34° *to* 42° *converts starch into glucose.* As pointed out elsewhere [Davis, 1916, p. 35], Brasse realised very clearly the instability of the glucose-forming enzyme in presence of alcohol and at temperatures above 40°. He did not, however, identify it as a maltase (*glucase*), which enzyme was discovered by Cuisinier [1885] in the same year.

That Brown and Morris did not recognise the presence of maltase in leaves may be explained by the fact that, although they carried out their transformations at 30°, they used leaves which had previously been dried at 40–50° and chloroform as an antiseptic; the drying of the leaves under the conditions named would probably destroy much of the maltase whilst *ordinary* impure chloroform also renders it inactive.

The results obtained by Vines [1891] call for a word of comment. Vines showed that his leaf extracts acted on starch paste giving a cupric-reducing and fermentable sugar which he concluded was not maltose because it had no optical activity. The explanation is that Vines worked with a very large amount of leaf material so that the substance he finally obtained was a mixture of the leaf-sugars (largely consisting of invert sugar) with the products of starch transformation; by chance the positive and negative rotations of the sugars present balanced so as to give an apparently inactive product.

Robertson, Irvine and Dobson [1909] have recently stated that they found maltase as well as ordinary diastase in the leaf and root of the sugar beet. From the results given later it is clear that maltase is also present abundantly in the leaf of the closely allied mangold. This fact is very striking when taken in conjunction with the complete absence of starch from the mangold leaf during all its later stages of growth [Davis, Daish and Sawyer, 1916].

The experiments which are given below show that all the plants we have examined, which include the *Tropæolum majus* investigated by Brown and Morris, contain maltase in considerable quantities; this is true whether the leaves be plucked in the daytime or at night.

EXPERIMENTAL.

To avoid destroying the maltase the freshly picked leaf material was always used without drying; for the same reason toluene was used as antiseptic, not chloroform, and the starch conversions were carried out at 38°, which is the optimum temperature for the maltase. The leaves were ground up in a mortar with a little water and the pulp was allowed to act on the starch paste or soluble starch during 24 hours or longer.

ACTION OF LEAF PULP ON SOLUBLE STARCH AT 38°.

Series I.

To 100 cc. of a soluble starch solution (= 2·2481 g. of anhydrous soluble starch dried *in vacuo* at 100° and having $[\alpha]_D^{20^\circ} = 191{\cdot}1^\circ$) 3 g. of wet leaf previously pulped in water were added; the volume was made up to 200 cc. and after adding a little toluene was digested at 38° for 24 hours. The mixture was then boiled to destroy the enzymes, 10 cc. of alumina cream were added and the volume diluted to 500 cc. at 15°. A control experiment was made with 3 g. of leaf under the same conditions, so as to allow for the sugars in the leaf. The results are given in Table I.

TABLE I.

Leaves plucked at 11 *a.m., September* 10, 1913.

Expt.	Leaf	CuO from 50 cc. of the 500 cc. (corr.)	$a_D^{20^\circ}$ in 200 mm. tube (corr.)	Total rotation of solution, reducing sugars calculated as	
				Maltose	Glucose
1	*Tropæolum majus*	0·1264	1·459°	1·559°	1·474°
2	Potato	0·0380	1·599°	1·668°	1·643°
3	Dahlia	0·1450	1·424°	1·536°	1·450°

The following example shows the method of calculation; the methods of analysis are those adopted by Davis and Daish [1913], the reducing power being determined under Brown, Morris and Millar's conditions.

(*a*) *Reducing sugars assumed to be entirely maltose:*

$$\text{Maltose in 50 cc.} = \frac{0{\cdot}1264}{1{\cdot}374} = 0{\cdot}0920 \text{ g.}$$

Therefore total maltose in 500 cc. = 0·920 g.

$$\text{Starch corresponding with this} = \frac{0{\cdot}920}{1{\cdot}055} = 0{\cdot}8717 \text{ g.}$$

Soluble starch taken = 2·2481 g.

Therefore starch remaining = 2·2481 – 0·8717 = 1·3764 g.

Rotation in 200 mm. tube, $a_D^{20^\circ}$ due to maltose = 0·507°

Rotation in 200 mm. tube due to soluble starch ($[\alpha]_D^{20^\circ} = 191{\cdot}1^\circ$) = 1·052°

Total rotation calculated = 1·559°

(*b*) *Reducing sugars assumed to be glucose:*

Glucose in 50 cc. $= \frac{0 \cdot 1264}{2 \cdot 578} = 0 \cdot 04903$.

Therefore total glucose in 500 cc. $= 0 \cdot 4903$ g. $\times\ 0 \cdot 9 = 0 \cdot 4413$ g. starch.

Therefore soluble starch remaining $= 2 \cdot 2481 - 0 \cdot 4413 = 1 \cdot 8068$ g.

Rotation due to glucose in 200 mm. tube ... $= 0 \cdot 093°$

Rotation due to soluble starch ... ... $= 1 \cdot 381°$

Total rotation $= 1 \cdot 474°$

In the case of *Tropæolum* and dahlia, which gave the highest proportion of reducing sugars and of which the diastatic activity was greatest, the rotation calculated on the assumption that the whole of the reducing sugar is glucose, is much closer to that actually observed than the value obtained assuming the sugar to be maltose. In these two cases the whole of the sugar appears to be glucose. In the case of the potato the diastatic action is far less and the calculation points to a mixture of maltose and glucose being present.

ANALYSIS BY MEANS OF MALTASE-FREE YEASTS.

Series II.

In order to make sure that glucose is formed from the soluble starch a series of experiments was carried out in which the product of transformation was analysed by means of the special yeasts (*S. marxianus* and *S. exiguus*) so as to obtain values showing the proportions of maltose and glucose actually formed [Davis and Daish, 1913].

50 cc. of a solution of soluble starch ($[\alpha]_D^{20°} = 192 \cdot 1°$), and corresponding with 1·0152 g. of soluble starch, were digested with 3 g. of wet leaf, after making the volume up to 200 cc., for 48 hours at 38° in the presence of toluene; at the end of this time 10 cc. of alumina cream were added and the mixture boiled and diluted to 500 cc. (solution *A*). For the fermentations, 150 cc. portions after adding 5 cc. of yeast-water were sterilised and inoculated with pure cultures of the yeasts. After 3 weeks, 5 cc. of alumina cream were added, the mixture was boiled and diluted to 200 cc. (solution *B*). 50 cc. portions were used for the cupric reductions (= 37·5 cc. of *A*) and the results obtained calculated back to 50 cc. of *A*.

In each experiment a duplicate control was made with 3 g. of the wet leaf so as to allow for the original sugars present in the leaf. The following table gives the corrected results.

Method of calculation: CuO from 50 cc. of A (corr. for leaf sugars) = 0·0888 g.
CuO due to maltose in 50 cc. of A = 0·0388 g.

CuO due to glucose in 50 cc. A = 0·0500 g., or $\frac{0{\cdot}0500}{2{\cdot}578} = 0{\cdot}0194$ g. glucose.

CuO due to maltose in 50 cc. A corresponds with $\frac{0{\cdot}0388}{1{\cdot}38} = 0{\cdot}0281$ g. maltose.

Total glucose in 500 cc. of A = 0·1940 g. = 0·1746 g. soluble starch
„ maltose „ = 0·2810 „ = 0·2664 „ „
Therefore total starch converted to sugars = 0·4410 g.
Total soluble starch taken = 1·0152 g.
Starch remaining in A = 1·0152 − 0·4410 = 0·5742 g.
Rotation of glucose in A, in 400 mm. tube ... ... = 0·082°
„ maltose „ „ ... ... = 0·310°
„ soluble starch in A in 400 mm. tube, taking $[\alpha]_D^{20^\circ} = 192{\cdot}1^\circ$ ... ... = 0·872°
Total = 1·264°
Actually observed in A = 1·222°

TABLE II.

Leaves picked at midnight, September 29, 1913.

Expt.	Leaf	CuO from 50 cc. of A (corr.)	CuO. corr. with 50 cc. A after fermentation			$\alpha_D^{20^\circ}$ of A in 400 mm. tube	$\alpha_D^{20^\circ}$ calc. for glucose, maltose and unchanged starch
			S. exiguus	*S. marxianus*	Mean		
4	*Tropæolum*	0·0382	—	—	—	1·385°	—
5	Dahlia	0·0888	0·0364	0·0412	0·0388	1·222°	1·264°
6	Mangold	0·0873	0·0462	0·0412	0·0437	1·218°	1·280°

In the case of the dahlia and mangold, these results clearly show that more than half of the sugar produced from the starch is glucose. In the case of *Tropæolum* the reducing power was so small that fermentations were not carried out. The results given in Table III however show that with this leaf the greater proportion of the sugar formed is glucose.

It is interesting to note that the diastatic power of the *Tropæolum* and dahlia leaves picked at midnight appears to be smaller than in the case of the same leaves picked at 11 a.m. (Table I). Using the same weight of leaf in the digestion, less reducing sugars have been formed and a smaller proportion of glucose in 48 hours than in the earlier results in 24 hours. As, however, no special precautions to ensure neutrality of the soluble starch were taken and as it is well known, from the work of Ford and Guthrie and of Sherman, that very small differences of reaction greatly influence the results obtained, no special significance can at present be attached to these data.

ACTION OF LEAF MATERIAL ON GELATINISED STARCH.

Series III.

In this series a known weight of starch dried *in vacuo* at 130° was gelatinised by boiling for 15 minutes with 200 cc. of water; the paste was digested with 5 g. of the wet leaf during 32 hours at 38°. The solution was then boiled and made up to 500 cc. at 15° (solution *C*). In these experiments, although nearly all of the starch was liquefied, a small quantity was left undissolved in every case; on filtering the solution it was found on the filter-paper. On this account it was not possible to compare the rotation of the solution obtained with that calculated for the sugars present. Fermentations were carried out as in Series II and controls made with 5 g. of the leaf material.

TABLE III.

Action of leaf enzymes on Gelatinised Starch. Leaves picked 12 midnight, October 3, 1913.

Expt.	Leaf	Vacuum dried starch taken	CuO from 50 cc. of *C* (corr.)	CuO from 50 cc. of *C* after fermentation	
				S. exiguus	*S. marxianus*
7	*Tropæolum*	2·2340	0·0493	0·0061	0·0100
8	Dahlia	2·3250	0·2170	0·1595	0·1655
9	Turnip	2·1418	0·2110	0·1308	0·1376

In these experiments the *Tropæolum* leaf again shows a relatively low diastatic activity as compared with that of the dahlia and turnip; the greater part of the sugar, however, is seen to be glucose. The turnip leaf is particularly active, which is interesting in view of the high proportion of starch which is characteristic of this leaf [Davis and Sawyer, 1916]. In all cases the leaf enzymes have produced a mixture of maltose and glucose, the maltose generally predominating.

ACTION OF LEAVES ON SOLUBLE STARCH.

Series IV.

100 cc. of a soluble starch solution (= 1·6855 g. vacuum dried soluble starch, having $[\alpha]_D^{20^\circ} = 193{\cdot}7^\circ$) were digested with 5 g. of wet leaf for 36 hours

at 38°. The solution was then boiled, 5 cc. of alumina cream were added and the volume made up to 500 cc. at 15° (solution *D*). Controls were made as usual. The calculations are similar to those of Series II.

TABLE IV.

Expt.	Leaf	CuO from 50 cc. solution *D*	CuO from 50 cc. *D* after fermentation		$\alpha_D^{20°}$ of solution *D*	
			S. exiguus	*S. marxianus*	Measured	Calc. for sugars and unchanged starch
10	Sunflower	0·0377	0·0123	—	0·612° (100 mm.)	0·612°
11	Dahlia	0·1449	0·0460	0·0532	1·065° (200 mm.)	1·060°

In both cases, especially in that of the dahlia which has the far higher diastatic activity, the larger part of the sugar consists of glucose. The agreement between the values observed and calculated for the mixture of sugars and unchanged soluble starch is very close.

SUMMARY.

1. It is shown that the crushed pulp of all the leaves examined (*Tropæolum*, potato, dahlia, turnip, sunflower and mangold) acts upon soluble starch or gelatinised starch, forming reducing sugars; of these the greater part consists of glucose, the rest being maltose. There is therefore no doubt as to the presence of maltase in these leaves, whether plucked at night or in the daytime.

2. When a relatively large excess of starch is used the conversion is generally incomplete. The action of the endo-cellular enzyme maltase being limited under these conditions of working, by its low solubility and low power of diffusion, maltose is nearly always found among the products.

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V. THE DISTRIBUTION OF MALTASE IN PLANTS. III. THE PRESENCE OF MALTASE IN GERMINATED BARLEY.

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It is still uncertain whether maltase, the enzyme which hydrolyses maltose to glucose, exists in germinated barley. Until recently no really satisfactory method existed of estimating glucose in presence of maltose and other sugars. Brown and Morris [1895] in a paper on the "isomaltose" of Lintner pointed out the danger of attempting to identify sugars solely by the properties of their osazones and their conclusions were supported by the results of Ling and Baker [1895, 1] with the same substance. The use of maltase-free yeasts, however, as suggested by Davis and Daish [1913] makes it possible accurately to estimate maltose when occurring with other sugars, and on this method the present work is based.

O'Sullivan [1872, 1876] showed that the sugar produced by the action of ordinary diastase on starch was not, as had generally been supposed, glucose, but maltose. Considerable confusion had in the past existed because the reducing powers of maltose and glucose were assumed to be identical; O'Sullivan showed that the reducing power of maltose is only 62 % that of glucose.

Brown and Heron [1879] also concluded that glucose is not formed by the prolonged action of malt extract on starch or maltose. It must be noted that in all their experiments the malt extract had either been heated before use to a temperature of 50° or the actual conversions were carried out at 55° or 60°. Brasse [1885], however, found that the diastase precipitated from an extract of germinated barley, providing the extraction is made rapidly in the cold and the alcohol used in precipitating the enzyme is not left too long in contact with it, digests raw starch grains, causing corrosion similar to that found during germination and producing at 32–42° not maltose, but glucose. At 50–57°, no glucose but only maltose was formed. It is clear from these facts that Brasse was working with a preparation containing maltase, but this enzyme was not then known; no experiments were made by Brasse to show whether his enzyme acted on maltose. To Cuisinier [1885, 1886] is due the credit of realising that a special enzyme, to which he gave the name *glucase*, is abundantly present in maize and that this leads to the formation of glucose; but he did not show that the glucase converts maltose into glucose nor that the glucose formed from starch is not split off directly from the latter. It was left to Geduld [1891], working in Cuisinier's distillery, to show that glucase hydrolysed maltose to glucose. Cuisinier specially states that "glucase" is present in the organs and particularly in the seeds of a great number of plants, whether they contain starch or not, a statement which has been repeatedly denied by later workers but is undoubtedly correct.

Lintner and Eckhardt [1889] concluded that extracts of raw, ungerminated barley gave with soluble starch the same products, namely maltose and dextrin, as were obtained with malt extract but the optimum temperature of action of barley diastase was considerably lower, viz. 45°, than that of malt diastase. Brown and Morris [1890] considered that maltose and dextrin only were formed by the action of the enzymes of either germinated or ungerminated barley on starch; they always used chloroform as antiseptic in their experiments. Lintner [1893] confirmed Cuisinier's statement that maize contained an enzyme capable of forming glucose from starch and stated that dried malt might sometimes give rise to not inconsiderable quantities of glucose. Ling and Baker [1895, 1, 2] also found a small amount of glucose to be formed by the action of the diastase of *kiln-dried* malt on starch at 70° and found that such diastase formed small quantities of glucose from maltose, the glucose being approximately estimated as osazone. When *low-dried* malt was used, no glucose was formed and they considered the kilning so to modify the diastase as to confer the property of converting starch into glucose. In a later paper [1897] they modify their views and state that kilning is not the origin of the glucose-forming enzyme. Their later experiments lead them to state that glucose is *never* formed by the action of normal malt on starch. Kröber [1895] on the contrary took the view that a "glucase" existed in germinated barley but was not present after kilning. Morris [1896], like earlier workers, found maltase in maize but no evidence of its presence in malted or unmalted barley; he states that "normal malt contains no enzyme capable of hydrolysing maltose."

Beyerinck [1895] whilst confirming the presence of "glucase" in maize denied its general distribution in plants as affirmed by Cuisinier. In wheat, rye and barley prior to germination no "glucase" could be found whilst during germination only very small quantities of glucase, if any, were formed. Beyerinck, as pointed out elsewhere [Davis, 1916], takes the view that "glucase" acts directly on *starch*, as well as on maltose, splitting off glucose directly.

Baker [1902] studying the action of the *precipitated* diastase of ungerminated barley on soluble starch found that after 6 hours at 50° only maltose and dextrins were formed; after 24 hours small quantities of glucose could be recognised by the osazone test. As glucose could not be detected after allowing this diastase to act on maltose but was formed by its action on "α-amylodextrin" he concluded that the glucose was formed from the latter and *not* from maltose. The action was regarded as not being due to maltase. At the ordinary temperature after 144 hours no glucose was formed from soluble starch, but only maltose and "α-amylodextrin." Ling and Baker [1902] found only maltose to be formed by the action of a diastase prepared from low-dried malt on starch paste at 50–60°. When the diastase was previously "restricted" by heating it above 65° its action was greatly modified and glucose was among the products of its long continued action on starch. B. F. Davis and Ling [1904] considered that the amount of glucose formed increased on raising the temperature of "restriction" until a maximum was reached at 65–70°. Glucose, however, was formed even when the enzyme had been restricted at 78°; but in this case the activity was greatly impaired. The maximum amount of glucose, estimated as osazone, was 12 % of the products of hydrolysis.

Marino and Sericano [1905] described the preparation from germinated barley dried at 25–30° of a "maltase" which hydrolysed maltose to glucose very vigorously. According to Marino and Fiorentino [1906] this "maltase" differs from the maltase of yeast in readily decomposing the β-glucosides and not the α-glucosides. In this respect this "maltase" resembled emulsin, but by a number of special experiments they considered they had shown that the property of decomposing the β-glucosides was not due to the presence of emulsin but to the "maltase" which hydrolysed maltose. No later work has been published to explain these abnormal results which contradict the generally accepted notions as to the action of maltase and the structure of maltose. Huerre [1909, 1, 2, 3, 4] studied the properties of the maltase prepared from different varieties of maize and buckwheat. From a consideration of the differences of optimum temperature and stability towards heat he considers that a whole family of "maltases" exists, the members of which have different solubilities and different resistances to heat.

Since the present work was undertaken Wierzchowski [1913, 1, 2] has published two papers in which he shows that not only is maltase present in maize but that it exists also in smaller quantities in all cereals, for example rye, barley, wheat, oats, buckwheat and millet. In his first paper he failed to find maltase in barley or barley diastase, but in his second paper succeeded in doing so. He clearly realised that the maltase is sparingly dissolved by water, and is easily destroyed by precipitation with ordinary alcohol. He therefore worked, as we have done, not with water extracts but with the ground meal from the grain, and at a low temperature (42–45°). Wierzchowski's view that the enzyme can form glucose directly from starch is discussed by Davis [1916].

The experiments described below confirm Wierzchowski's view that germinated barley contains maltase. As it was possible to raise the objection to Wierzchowski and earlier workers whose results pointed to the presence of maltase, that the latter arose from moulds or fungi present on the grain, special precautions were taken to work with seeds which had been sterilised beforehand. To Dr H. B. Hutchinson thanks are due for valuable help. The grain was sterilised by a short immersion in a solution of mercuric chloride; after washing thoroughly with sterilised water it was allowed to germinate in sterile Petri dishes in which each grain could be observed. In no case could the growth of any mould or foreign organism be detected. The action of the germinated grain after drying and grinding was tested directly on starch and maltose at 38° in presence of toluene as antiseptic.

The results which are given show that gelatinised starch is acted on by the ground meal, the cupric reducing power of the product increasing as time proceeds whilst the rotatory power falls. The fermentations with maltase-free yeasts show that maltose and glucose are both formed, the latter increasing with time, whilst the maltose correspondingly falls. Control experiments carried out by digesting the barley alone, in which case self-digestion of the starch of the grain occurred, show that after prolonged digestion practically all the starch is converted into glucose; but in the earlier stages, considerable proportions of maltose, as well as some dextrin, are present. When an excess of starch is used, as the maltase is relatively deficient, considerable amounts of maltose are found, but the latter is gradually transformed into glucose as time proceeds.

The results obtained with maltose show clearly that the germinated grain contains a true maltase. The action of the grain on starch is strictly comparable with that of taka-diastase [Davis and Daish, 1914; Davis, 1914] and the degradation of the starch by the enzymes of the germinated barley occurs in successive stages:

Starch ⟶ soluble starch ⟶ dextrins ⟶ maltose ⟶ glucose

[see Davis, 1916].

EXPERIMENTAL.

SERIES I. *Preliminary experiments with Unsterilised Barley.*

(i) *Self-digestion of germinated barley.* A sample of good barley, free from damaged grains, was soaked for two days in several changes of water. The seeds were then allowed to germinate in a heap on a cement floor until the rootlets were two to three times as long as the grain, the temperature being kept at 15–20°. The germinated material was dried in the air at 25–30°. Portions were then ground up in a mortar and allowed to digest in presence of 200 cc. of water at 38° for 24 hours, toluene being added (1 to 2 cc.) and the mixture well stirred at intervals. After the action the solution was boiled to destroy the enzymes, 10 cc. of alumina cream were added and the volume made up to 500 cc. at 15° (solution *A*). The weights of germinated barley represent the weights of vacuum-dried material. Air-dried material was weighed out but the weights have been reduced by the amount of water found to be present when similar material was dried to constant weight *in vacuo* at 130°. The percentage of water present was 13·2 %.

The analyses were made by the methods described by Davis and Daish [1913], the cupric reducing powers being determined under Brown, Morris and Millar's standard conditions. The values given in each case are the mean of two closely concordant results. All weights are in grams. The rotations are in true angular degrees.

For comparison of the results obtained on digestion of the barley by its own enzymes with those given by taka-diastase digestion, a similar quantity of the ground barley was heated with boiling water (200 cc.) to gelatinise the starch and destroy the enzymes. After cooling to 38°, 0·1 g.

TABLE I.

Self-digestion of Germinated Barley at 38° (24 hours).

Expt.	Weight of vacuum-dried germinated barley	CuO from 50 cc. of *A*	$\alpha_D^{20^\circ}$ of *A* in 400 mm. tube	$\alpha_D^{20^\circ}$ calc. for reducing sugars as maltose	$\alpha_D^{20^\circ}$ calc. for reducing sugars as glucose
1	4·0933	0·2605	1·076°	2·108°	0·434°
2	4·5561	0·4287	2·696°	3·475°	0·756°

of taka-diastase was added and the mixture digested 24 hours at 38°. The solution was then treated as above, 10 cc. of alumina cream added and the volume made up to 500 cc. (solution *B*). The results in Table II are corrected for the 0·1 g. of taka-diastase by a control experiment under similar conditions.

TABLE II.

Digestion of Germinated Barley with 0·1 *g. Taka-diastase.*

Expt.	Weight of vacuum-dried barley	CuO from 50 cc. of *B*	$\alpha_D^{20^\circ}$ of solution *B* in 400 mm. tube	$\alpha_D^{20^\circ}$ calc. for reducing sugars as maltose	$\alpha_D^{20^\circ}$ calc. for reducing sugars as glucose
3	4·0937	0·4485	1·899°	3·633°	0·798°
4	4·4948	0·4842	2·093°	3·923°	0·873°

The results in Tables I and II show that the digestion of the barley by its own enzymes produces nearly the same result as that effected by taka-diastase, which contains maltase, after destroying the barley enzymes. In all cases a mixture of maltose and glucose is formed; the rotations observed are roughly the mean of those calculated for the two sugars, showing that nearly equal quantities of these were produced in 24 hours. In the case of the self-digestion of the barley it should be noted that the starch was not previously gelatinised so that in this case the action took place on the starch granules.

In the following experiments the maltose was estimated by the use of maltase-free yeasts so that the proportion of the two sugars could be calculated.

(ii) *Estimation of maltose and glucose formed by the self-digestion of germinated barley and by the action of the barley on starch.* Approximately 5 g. of the germinated barley was ground up and digested in presence of 200 cc. of water (*a*) with 2·1210 g. of vacuum-dried potato starch, after gelatinising the latter; (*b*) without adding any starch. The digestions were continued for 48 hours at 38°, sufficient toluene being added. The solution was then boiled, 10 cc. of alumina cream being added, and the mixture diluted to 500 cc. at 15° (solution C). After determining the reducing and rotatory powers, 100 cc. portions of the solution were sterilised by heating in the autoclave, after adding 5 cc. of yeast water, and fermented in the usual way with pure cultures of *S. exiguus* and *S. marxianus* for 28 days. The solutions were then boiled and 5 cc. of alumina cream added. Owing to the evaporation which had taken place in the course of the month the total volume was now slightly below 100 cc. and could be adjusted exactly to 100 cc. by adding water (solution D). 50 cc. portions of D were used to measure the cupric reducing power.

TABLE III.

Sugars formed by the Self-digestion of 5 *g. of Germinated Barley and by its action on Gelatinised Starch at* 38° (48 *hours*).

Expt.	Conditions	CuO from 25 cc. of C	CuO after fermentation from 50 cc. $D = 50$ cc. solution C		Total maltose in 500 cc. C	Total glucose in 500 cc. C
			S. exiguus	*S. marxianus*		
5	5 g. ground germinated barley + 2·1210 vacuum-dried starch	0·2938	0·2590	0·2574	1·892	1·300
6	5 g. germinated barley only	0·1194	0·0508	0·0526	0·3746	0·7256

Expt.	Conditions	$\frac{\text{Glucose}}{\text{Maltose}}$	$\alpha^{20°}_D$ observed in C (400 mm.)	$\alpha^{20°}_D$ calc. for maltose + glucose in C	Total starch from sugars[1]	Percentage of starch in barley (air-dried) digested to sugars
5	5 g. ground germinated barley + 2·1210 vacuum-dried starch	0·69	2·700°	2·631°	2·964	14·9
6	5 g. germinated barley only	1·93	0·637°	0·565°	1·008	20·2

In Exp. 5, where the germinated barley has acted on gelatinised starch, far more reducing sugars (3·192 g.) have been formed than from the 5 g. of barley alone (1·100 g.). On the other hand, in the first case the greater part of the sugar is in the form of maltose (glucose/maltose = 0·69) whilst in the second it is present as glucose (glucose/maltose = 1·93). Whilst the grain contains sufficient maltase to convert the greater part (two-thirds) of its own starch into glucose, when an excess of starch has been added more maltose is formed than can be dealt with by the limited amount of maltase present. The liquefying and maltose-forming enzymes are in relative excess as compared with the maltase, so that far more maltose is formed from the excess of starch than can be transformed into glucose.

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[1] In calculating these results the sugars originally present in the germinated barley have been neglected. Strictly these are by no means negligible as the results in Section v show, but for the purposes of *comparison* and the argument of the present paper they can be ignored. The results for starch given are in reality higher than the true values by the amount of pre-existent sugars.

In Exp. 5 the sugars formed correspond with considerably more starch (2·964 g.) than that taken (2·1210 g.), owing to the fact that the starch of the grain has been partly digested. It is probable that the whole of the gelatinised starch has been converted into sugars in the 48 hours and that the starch of the grain is undergoing a slower conversion, owing to its granular nature. This would explain the fact that the excess of the rotation observed over that calculated for the sugars, which in Exp. 5 is + 0·069° and in Exp. 6 + 0·072°, is practically identical in the two cases. This excess no doubt is due to the presence of a nearly constant quantity of dextrin in both cases: the amount of this dextrin is relatively very small, as compared with the sugars, the dextrin being transformed into sugars almost as fast as it is produced from the starch. If we calculate in Exp. 5 the quantity of starch *of the grain* which has been converted into sugars by assuming that the whole of the gelatinised starch has disappeared, it amounts to 14·9 % of the weight of the barley taken. In Exp. 6, the digested starch represents 20·2 % of the barley, showing that the addition of the gelatinised starch has arrested to some extent the self-digestion of the grain itself. The barley probably contains some 50 % of starch (see Table VII), so that in 48 hours only a small proportion of the total has been digested.

(iii) *Action of small quantities of germinated barley on soluble starch.* When small quantities of germinated barley (0·1 and 0·2 g.) are added to soluble starch considerably less conversion of the starch occurs than in Exp. 5 where 5 g. were used. After 36 hours, more than half of the soluble starch is left in the form of dextrin, whilst the greater part of the sugar remains in the form of maltose, insufficient maltase being present to effect anything like complete transformation of the latter.

In these experiments 2·17 g. of vacuum-dried soluble starch were digested with 0·1 and 0·2 g. of powdered germinated barley for 36 hours at 38°; 5 cc. of alumina cream were then added, and, after boiling, the volume was made up to 500 cc. (solution *E*). Portions of 100 cc. after adding 5 cc. of yeast water were fermented for 28 days, as in the earlier experiments, and finally made up exactly to 100 cc.

The values given show that after 36 hours' digestion 44·3 % of the soluble starch has been converted into sugars by 0·1 g. of germinated barley, and 56·3 % by 0·2 g. of the latter. In both cases, nearly the whole of the sugar is in the form of *maltose*, the ratio of glucose to maltose in the two experiments being 0·037 and 0·077 respectively. The use of the doubled quantity of barley has just doubled the ratio. The large differences between the rotations actually observed and those calculated for the sugars are due to unconverted soluble starch, as shown by the values in the last column. The differences (Δ) are strictly proportional to the starch unconverted: thus

TABLE IV.

Conversion of Soluble Starch by small quantities (0·1–0·2 *g.*) *of Germinated Barley* (36 *hours at* 38°).

Expt.	Conditions	CuO from 50 cc. solution E	CuO from 50 cc. solution E after fermentation by		Total maltose in 500 cc. E	Total glucose in 500 cc. E	Glucose / Maltose
			S. exiguus	*S. marxianus*			
7	2·17 g. soluble starch + 0·1 g. germinated barley	0·1443	0·1348	0·1349	0·9785	0·0368	0·037
8	2·17 g. soluble starch + 0·2 g. barley	0·1882	—	0·1644	1·200	0·0923	0·077

Expt.	Conditions	Percentage of starch taken converted to sugars	$\alpha_D^{20^\circ}$ of solution E (400 mm.)	$\alpha_D^{20^\circ}$ calculated for sugars only	Δ	$\alpha_D^{20^\circ}$ calc. for sugars + unaltered soluble starch[1]
7	2·17 g. soluble starch + 0·1 g. germinated barley	44·3	2·874°	1·092°	1·782°	2·967°
8	2·17 g. soluble starch + 0·2 g. barley	56·3	2·805°	1·360°	1·445°	2·831°

$$\frac{1\cdot782}{1\cdot445} = 1\cdot233\,; \quad \text{and} \quad \frac{100 - 44\cdot3}{100 - 56\cdot3} = \frac{55\cdot7}{43\cdot7} = 1\cdot274.$$

(iv) *Attempt to separate the maltase from the germinated barley by extraction and precipitation.* Two attempts were made to separate an active preparation of maltase from the germinated barley. For this purpose a method similar to that stated to be successful by Marino and Fiorentino [1906] was used.

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[1] The soluble starch used had $[\alpha]_D^{20^\circ} = 193\cdot7^\circ$ and this value has been used in the calculation for the total rotation of the solution due to maltose, dextrose and unchanged soluble starch. The values actually observed are very slightly lower than those calculated. In presence of the excess of soluble starch it would appear therefore that practically none of the starch of the added grain has passed into solution. No correction has been made for the small amount of soluble sugars in the added grain.

The powdered *air-dried* grain was extracted with 1·5 times its weight of water saturated with thymol, the extract filtered and the solid matter pressed to remove as much liquid as possible. The extract was slowly dropped from a dropping funnel into eight to nine times its volume of 95 % alcohol, kept rapidly stirred. A large quantity of a slightly rose-coloured precipitate was obtained; this was immediately filtered off under pressure and dried in a vacuum desiccator. It was then redissolved in the smallest quantity of water saturated with thymol and again precipitated by dropping into alcohol. After filtering rapidly the precipitate was again dried *in vacuo*. The preparation so obtained acted very rapidly on soluble starch, but gave only maltose and dextrin as products, and no glucose. A second preparation gave a similar result. It is clear that either the method of extraction failed to dissolve the maltase or the latter was destroyed in the subsequent operations. The method described differs from that of Marino and Fiorentino in that the latter concentrated the extract *in vacuo* at 35–40° before precipitating with alcohol. They also finally dialysed their product. It is probable that differences in the nature of the alcohol may explain the differences between our results and those of Marino and Fiorentino; similar differences are to be found between the results of Beyerinck and Wierzchowski as regards the maltase of maize [see Davis, 1916, p. 34]. In any case, the negative results obtained show the instability of the maltase and the difficulty of isolating it.

SERIES II. *Experiments with Sterilised Barley.*

(v) *Analysis of the sugars present in germinated barley.* Two hundred barley grains (weight = 8·4223 g.) were sterilised in mercuric chloride solution and, after washing with sterilised water, were soaked for three days and allowed to germinate separately in sterile Petri dishes for a period of seven days. They were then dried in air at 25–30° and after grinding in a mortar were extracted in a Soxhlet extractor with 80 % alcohol for 18 hours. The extract was evaporated *in vacuo* at 35° and after the addition of 2 cc. of basic lead acetate solution made up to 100 cc. (solution *F*). 20 cc. portions were used to estimate the reducing sugars and saccharose (by inversion with 10 % citric acid); two further portions were fermented with *S. marxianus* and *S. exiguus* to give the maltose.

The values given, which must be regarded as only preliminary, show that maltose and glucose are present in nearly equal proportions; a slightly smaller amount of saccharose is present. The maltose was calculated as usual from the reducing power left after fermentation; the difference of reducing power, 0·3149–0·1127 CuO, was assumed to be due to glucose. The sum of the rotations, 1·924°, due to the three sugars, assuming the whole of the reducing sugar other than maltose to be glucose, is slightly *less* than the value actually observed. This excludes the possibility that the reducing sugars consist largely of invert sugar. If the rotation be calculated for a mixture of maltose, invert sugar and saccharose it becomes + 1·325° which is much further removed from the value observed (2·265°) than on the assumption that glucose is present. It is probable therefore that the sugars are maltose, glucose and saccharose, with a trace of dextrin sufficient to cause the difference between the observed and calculated values.

TABLE V.

Sugars present in Germinated Barley.

				CuO from 20 cc. F after fermentation with		
Expt.	Weight of air-dried barley	CuO from 20 cc. of solution F	CuO from 20 cc. F after inversion	*S. marxianus*	*S. exiguus*	Maltose percentage on barley
9	8·4223	0·3149	0·4456	0·1117	0·1137	4·91

Expt.	Glucose percentage on barley	Saccharose percentage on barley	$a_D^{20°}$ of F in 200 mm. tube	$a_D^{20°}$ calc. for mixture of sugars in F	Total sugars percentage in barley
9	4·80	3·21	2·265°	1·924°	12·92

(vi) *Action of germinated barley on gelatinised starch.* The sterilised barley was allowed to germinate in Petri dishes until the plumules were about ¼″ long. The grains were then removed, dried *in vacuo* over sulphuric acid and ground to a fine meal. About 1 g. of potato starch (vacuum-dried at 130°) was gelatinised by heating for 15 minutes with 50 cc. of water in a 150 cc. Jena flask and, after cooling to 38°, 0·2 g. of the barley meal was added and the mixture left to digest for various lengths of time, in presence of sufficient toluene to prevent any growth of micro-organisms, the flask being closed with a plug of cotton wool. After the digestion in each case 10 cc. of alumina cream were added, the solution raised to the boil, filtered and washed to 100 cc. at 15° (solution G).

20 cc. portions were used to determine the reducing power. 20 cc. portions were also mixed with 5 cc. of yeast water and fermented with the special yeasts for 21 to 25 days; after the fermentation 5 cc. of alumina cream were added, the mixture being boiled and filtered. The precipitate was washed until the volume of the filtrate was 50 cc., the whole of the latter being used for the reduction.

These results clearly show that as time proceeds the maltose present decreases in amount whilst the glucose increases. The ratio of glucose to

TABLE VI.

Action of Germinated Barley (0·2 *g.*) *on Gelatinised Starch.*

Expt.	Time of digestion hours	Weight of vacuum-dried starch	CuO from 20 cc. of G	CuO from 20 cc. of G after fermentation with *S. marxianus*	*S. exiguus*	Mean	Total maltose in 100 cc. of G
10	119	1·0532	0·3973	0·1598	0·1608	0·1603	0·5885
11	191	1·0192	0·4250	0·1458	0·1398	0·1428	0·5240
12	263	1·0468	0·4551	0·1363	0·1342	0·1353	0·4967

Expt.	Total glucose in 100 cc. of G	$\frac{\text{Glucose}}{\text{Maltose}}$	Total starch from sugars	Starch found as percentage of starch taken	$\alpha_D^{20°}$ of G in 400 mm. tube	$\alpha_D^{20°}$ of sugars of G in 400 mm. tube	Δ
10	0·4866	0·827	0·9959	94·6	5·443°	4·266°	+1·177°
11	0·5885	1·125	1·0263	100·7	4·838°	4·125°	+0·713°
12	0·6770	1·363	1·0802	103·1	4·691°	4·162°	+0·529°

maltose increases from 0·827 after 119 hours to 1·125 after 191 hours and to 1·363 after 263 hours. During the whole period part of the starch is present in solution in the form of dextrin as is shown by the value of $\alpha_D^{20°}$ observed being greater than that calculated from the glucose and maltose present. As time goes on, this difference, Δ, diminishes, being 1·177°, 0·713° and 0·529° at the different times of digestion, but even after 263 hours some dextrin is still present. The amount of enzyme taken in the 0·2 g. of barley is so small that conversion of the total starch into sugars has not been complete even after 263 hours. The action is not, however, at a standstill even after this prolonged period, as shown by the steadily increasing values of the glucose and total starch and the falling values of the maltose. The fact that the ungelatinised starch of the barley is being slowly converted into sugars accounts for the values found at the 191st and 263rd hours slightly exceeding 100 % of the gelatinised starch taken. It is noteworthy that during the last stages the increase of the glucose is greater than the corresponding fall of maltose in the same period, probably owing [see Davis, 1916, p. 42] to the rate of formation of maltose from the dextrin in solution being greater than that of the formation of glucose from maltose.

It is possible to calculate approximately at each interval the amount of the starch of the grain which has been converted into a soluble form. If this be done it is found that after the 119th hour, 33·5 % of the total weight of the added barley has been digested, after the 191st hour 37·2 %, and after the 263rd hour 38·9 %. As the actual starch present in the grain is probably about 50 %, some starch still remains undigested even after so long a period of action. For the purpose of control, experiments were made to ascertain the degree of self-digestion of 0·2 g. of the germinated barley under exactly the same conditions as those of the experiments given in Table VI. The results are given in Table VII (solutions obtained in these controls are designated *G′*).

It is necessary to make allowance for the pre-existent sugars in the germinated barley. The method of calculation is as follows: the values for the original sugars given in Table V are used.

In 400 mm. tube

Maltose originally present in 0·2 g. barley =0·0098 producing $\alpha_D^{20°} = +0{\cdot}054°$

Glucose " " " " =0·0096 " $= +0{\cdot}020°$

Saccharose converted into invert sugar in 0·2 g. barley =0·0061 " $= -0{\cdot}005°$

Therefore maltose actually produced from starch

=0·5885 − 0·0098 = 0·5787 g. with rotation = +3·185°

Therefore glucose actually produced from starch

=0·4866 − 0·0096 − 0·0061 = 0·4709 g. with rotation = +0·993°

Starch corresponding to maltose = 0·5787 ÷ 1·055 = 0·5485 g.

" " glucose = 0·4709 × 0·9 = 0·4238 g.

Total starch = 0·9723 g.

Gelatinised starch not converted to sugars = 1·0532 − 0·9723 = 0·0809 g.

If this is in solution as dextrin with $[\alpha]_D^{20°} = +202°$, it produces rotation = +0·654°

Total rotation from above constituents ... = +4·901°

Actually observed rotation ... ... = +5·443°

$\Delta = +0{\cdot}542°$

If Δ is due to the starch of the grain, transformed to dextrin (or soluble starch), with $[\alpha]_D^{20°} = 202°$, the concentration of the latter in 100 cc. of solution = **0·0671** g., which on 0·2 g. of germinated barley = **33·5** %.

The following table gives a summary of the results calculated in this way for the three periods of digestion.

Time in hours	Δ° due to dextrin in solution *G*	Dextrin arising from grain in g.	Percentage of barley transformed
119	+0·542	0·0671	33·5
191	+0·601	0·0744	37·2
263	+0·548	0·0678	38·9

At the 263rd hour more than the whole of the gelatinised starch taken has been converted into sugars and 0·0099 g. of the starch of the barley has passed into the form of sugars. Hence the falling off in the value of Δ from the 191st to 263rd hour.

TABLE VII.

Self-digestion of 0·2 *g. of Germinated Barley.*

Expt.	Time in hours	CuO from 20 cc. of *G′*	CuO from 20 cc. *G′* after fermentation with *S. marxianus*	*S. exiguus*	Mean	Total maltose in 100 cc. *G′*	Total glucose in 100 cc. *G′*
10′	119	0·0506	0·0046	0·0034	0·0040	0·0145	0·0903
11′	191	0·0570	0·0034	0·0010	0·0022	0·00795	0·1062
12′	263	0·0607	—	0·0032	0·0032	0·0116	0·1114

Expt.	$\frac{\text{Glucose}}{\text{Maltose}}$	$\alpha_D^{20°}$ observed in 400 mm. tube	$\alpha_D^{20°}$ calculated for sugars	Δ	Starch corresponding with sugars	Percentage starch in barley
10′	6·2	0·345°	0·170°	+0·175°	0·0950	47·5
11′	13·4	0·241°	0·268°	−0·027°	0·1032	51·6
12′	9·6	0·255°	0·299°	−0·044°	0·1113	55·6

The above results show that the self-digestion of the barley by its own enzymes gives rise almost entirely to glucose when the digestion is allowed to proceed for a long period. On comparing these results with those of Table III and Table XII obtained with 5 g. of germinated barley it will be seen that whereas after 48 hours the ratio of glucose to maltose is 1·95, after 119 hours the maltose has practically disappeared, the ratio being 6·2; after 191 hours the ratio is 13·4 and after 263 hours 9·6. In Exp. 10′ some of the starch is still present as dextrin, $\alpha_D^{20^\circ}$ observed being greater than $\alpha_D^{20^\circ}$ calculated for the sugars ($\Delta = +\ 0{\cdot}175^\circ$). In Exps. 11′ and 12′ this dextrin has entirely disappeared and the values of Δ are slightly negative ($-\ 0{\cdot}027^\circ$ and $-\ 0{\cdot}044^\circ$). This is probably due to a small proportion of the reducing sugars consisting of laevulose, which has been formed by the inversion of the saccharose present in the original grain (see Table V).

The digestion of the grain is not complete after 119 hours and is still progressing slowly even after 263 hours. The sugars formed correspond with 47·5 % of starch after 119 hours, with 51·6 % after 191 hours and with 55·6 % after 263 hours. These values for starch are probably higher than the true starch in the grain as they include the original sugars present, which amount to 12·92 % (see Table V).

If we use the values given in Table VII as a "control" to correct the values for the digestion of gelatinised starch by 0·2 g. of germinated barley we obtain the following data.

TABLE VIII.

Action of 0·2 g. Germinated Barley on Gelatinised Starch corrected for the Barley used.

Time in hours	CuO from 20 cc.	Maltose CuO (corr.) from 20 cc.	Glucose CuO (corr.) from 20 cc.	Total maltose in 100 cc.	Total glucose in 100 cc.	Glucose / Maltose
119	0·3467	0·1563	0·1904	0·5745	0·3926	0·68
191	0·3680	0·1406	0·2274	0·5150	0·4641	0·90
263	0·3944	0·1321	0·2623	0·4849	0·5550	1·14

Time in hours	Total starch calc. from sugars	Starch as percentage of gelatinised starch taken	$\alpha_D^{20^\circ}$ (corr.) observed in 400 mm. tube	$\alpha_D^{20^\circ}$ calc. for sugars	Δ
119	0·8982	85·3	5·098°	3·990°	1·108°
191	0·9059	88·9	4·597°	3·814°	0·783°
263	0·9591	91·6	4·436°	3·838°	0·598°

The "corrected" values show again that as time proceeds the proportion of glucose increases whilst that of maltose falls; the ratio glucose : maltose which after 119 hours is 0·68 rises to 0·90 after 191 hours and to 1·14 after 263 hours. The proportion of starch converted into sugars rises from 85·3 % after 119 hours to 88·9 % and 91·6 % after 191 and 263 hours respectively. The difference Δ between the values of $\alpha_D^{20^\circ}$ observed and calculated for the sugars gradually falls from 1·108° at the 119th hour to 0·783° and 0·598° at the later times, as the dextrin in solution disappears owing to its transformation into sugars.

It is probable that actually the whole or the greater part of the gelatinised starch is converted into sugars, as suggested by the data of Table VI, before the more resistant starch granules of the barley are dissolved by its own enzymes, and that the latter change only makes marked progress when the gelatinised starch has largely disappeared. The self-digestion of the barley is in fact greatly modified in the earlier stages by the presence of the excess of starch so that it is incorrect to apply as a "correction" the values given in Table VII for the self-digestions carried out with the grain alone. As the gelatinised starch is transformed into sugars, the self-digestion of the added barley probably proceeds more rapidly but there is no means of ascertaining what proportion of the two kinds of starch present should figure in the calculations at the different intervals of time. As will be seen later, the self-digestion of the "controls" is greatly modified by the presence of an excess of maltose. In the latter case the effect can be more clearly traced (see p. 72).

(vii) *Action of* 0·2 *g. of germinated barley on maltose at* 38°. The values given above showing the transformation of starch suggest that the glucose is produced from pre-formed maltose by the action of a maltase. To test this point the action of the germinated grain on maltose was studied.

The same barley was used as in the experiments on starch. An approximately 5 % solution of maltose (recrystallised several times from 80 % alcohol) was used. 20 cc. portions of this were digested with 30 cc. of water and 0·2 g. of the finely ground barley as in the experiments with starch in presence of sufficient toluene to prevent the growth of micro-organisms. At the end of the digestion 10 cc. of alumina cream were added to each flask and the contents were boiled to destroy the enzymes. After filtering, the precipitate was washed until the volume of the filtrate was nearly 100 cc., and the solution was finally made up exactly to 100 cc. at 15° (solution *H*). 20 cc. portions of the filtrate were used to determine the reducing power and 20 cc. portions were fermented with *S. marxianus* and *S. exiguus* as in the starch experiments. 20 cc. of the original maltose solution were diluted to 100 cc. and the reducing power and rotatory power determined with 20 cc. portions (+ 30 cc. water) so as to obtain values comparable with those of the digestions. That prolonged digestion with water alone has no action on maltose was shown by the following experiment. 1·006 g. of the maltose was added to 50 cc. of water and after adding 5 cc. of toluene the mixture was left in a flask closed with cotton wool for 165 hours at 38°. 5 cc. of alumina cream were added, the solution was boiled and made up to 100 cc. at 15°. The rotation of the original solution was 5·290° in a 400 mm. tube at 20°; after the prolonged digestion and treatment as above, the rotation was 5·291°.

TABLE IX.

Action of Germinated Barley (0·2 *g.*) *on approximately* 1 *g. Maltose in* 50 *cc. of water at* 38°.

			CuO from 20 cc. of solution *H* after fermentation with				
Expt.	Time in hours	CuO from 20 cc. of solution *H*	*S. marxianus*	*S. exiguus*	Mean	Maltose in 100 cc. *H*	Glucose in 100 cc. *H*
13	Nil	0·2722	—	—	—	0·9980	—
14	119	0·3672	0·1580	0·1585	0·1583	0·5810	0·4268
15	191	0·4006	0·1184	0·1247	0·1216	0·4463	0·5765
16	263	0·4262	—	0·1048	0·1048	0·3848	0·6710

				$\alpha_D^{20^\circ}$ of solution *H* in 400 mm. tube		
Expt.	$\frac{\text{Glucose}}{\text{Maltose}}$	Total sugars in solution calc. as maltose	Percentage of maltose left unchanged	Observed	Calc. from sugars	Δ
13	—	0·9980	100·0	5·300°	—	—
14	0·735	0·9860	58·2	4·403°	4·098°	+0·305°
15	1·292	0·9939	44·8	4·035°	3·671°	+0·364°
16	1·744	1·0223	38·6	3·763°	3·533°	+0·230°

If we "correct" these values by the control experiments carried out with 0·2 g. of germinated barley (Table VII) we obtain the following data (Table X).

The results given in Table IX are very striking. The values for maltose and glucose are calculated from the reductions before and after fermentation in the usual way. The maltose is seen to fall steadily whilst the glucose increases. The ratio glucose : maltose rises steadily from 0·735 after 119 hours to 1·744 after 263 hours. From the values given in the 10th column for the total maltose corresponding with the sugars in solution[1] it is seen that during nearly the whole of the action these sugars correspond only with the maltose taken; practically no sugar has been formed from the starch of the barley. *Thus in presence of the excess of maltose the self-digestion of the barley, which gives rise to the values in Table VII, has been almost entirely suppressed.* It is only in the last stage of the action (263rd hour) that the total sugars expressed as maltose (1·0223 g.) exceed the maltose originally taken (0·9980 g.); even here the excess is small, but there is no doubt that in these latter stages additional sugar is being formed from the barley.

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[1] Calculated as follows, for example for the 119th hour:
Total sugars as maltose = 0·5810 g. + (0·95 × 0·4268) = 0·9860 g.

TABLE X.

Hydrolysis of Maltose by 0·2 g. Germinated Barley, "corrected" for the Barley used.

Expt.	Time in hours	CuO from 20 cc. H' (corr.)	Glucose CuO from 20 cc. of H'	Maltose CuO from 20 cc. of H'	Glucose in 100 cc. of H'	Maltose in 100 cc. of H'
13′	Nil	0·2722	—	0·1996	—	0·9980
14′	119	0·3166	0·1623	0·1543	0·3315	0·5650
15′	191	0·3436	0·2242	0·1194	0·4630	0·4383
16′	263	0·3655	0·2639	0·1016	0·5510	0·3730

Expt.	$\frac{\text{Glucose}}{\text{Maltose}}$	Percentage of maltose left unchanged	$\alpha_D^{20^\circ}$ (corr.) in 400 mm. tube			Total sugars calc. as maltose
			Observed	Calc. for sugars	Δ	
13′	—	100·0	5·300°	—	—	0·9980
14′	0·586	56·7	4·058°	3·809°	+0·249°	0·8799
15′	1·057	43·9	3·794°	3·389°	+0·405°	0·8781
16′	1·478	37·4	3·508°	3·215°	+0·293°	0·8955

A comparison of the values of $\alpha_D^{20^\circ}$ calculated for the sugars with those actually observed shows that up to the 191st hour the observed rotation is in excess of that calculated by a *nearly constant* but slightly increasing amount (+ 0·305° to + 0·364°); this is no doubt due to the formation of dextrin from the starch of the 0·2 g. of barley, the dextrin increasing only slightly in amount and undergoing very little if any transformation into maltose in the earlier stages. During the last stage of the action (191st to 263rd hour), when the concentration of the original maltose has greatly fallen, the dextrin from the barley starch apparently begins to be transformed into maltose, as shown by the value of Δ diminishing from 0·364° to 0·230°, whilst the total sugars expressed as maltose slightly exceed the original maltose taken.

The above results show that when working with enzymic material, such as germinated barley, a control experiment carried out with this material and water alone does not always give values which can be applied as a true correction, owing to the activity of the enzyme being greatly modified by the presence of the substrate the change of which is being studied. From Table X it is seen that whilst the maltose values are only slightly changed by introducing a "correction" for the 0·2 g. of barley, since the maltose values obtained with the latter are in themselves very small, the results for glucose are very different from those in Table IX, and far lower than they should be. The sum of the sugars calculated as maltose is always far less than the original maltose taken (viz. 0·8781 to 0·8955 instead of 0·9980), the difference being greatest in the early stages of the action when the effect of the maltose in limiting the self-digestion of the starch is most pronounced. It is clear that the "correction" introduced in this way is far greater than that corresponding with the actual self-digestion of the starch under the modified conditions of experiment.

The formation of glucose from maltose takes place even in short times of digestion. The following series of experiments was carried out to ascertain whether glucose can be detected in the earliest stages of the action. The details of the experiments were the same as in those described above but the times of digestion were shorter.

TABLE XI.

Action of 0·2 *g. Germinated Barley on Maltose Solution at* 38°.

Expt.	Time of digestion hours	Mean CuO from 20 cc. of solution	$\alpha_D^{20^\circ}$ observed in 400 mm. tube
17	Nil	0·2746	5·36°
18	24	0·2857	4·98°
19	48	0·2927	4·92°
20	145	0·3395	4·04°

In these experiments no fermentations were made to ascertain the proportion of maltose and glucose present. It is clear, however, from the increase of reducing power and the fall of rotatory power that the glucose is formed from the start. Fig. 1 shows the result obtained in plotting the values of CuO and $\alpha_D^{20^\circ}$; both the reducing power and the polarisation appear as linear functions of the time.

(viii) *Self-digestion of* 5 *g. of germinated barley.* 5 g. of the barley were ground up and digested at 38° with 100 cc. of water in presence of toluene during 48 hours. 10 cc. of alumina cream were then added, the solution was raised to the boil, filtered and made up to 500 cc. (solution K); 50 cc. portions were used for the direct measurements of cupric reducing power and similar portions were fermented as usual with the special yeasts.

After 48 hours' self-digestion of the barley at 38° roughly two-thirds of the sugar in solution are in the form of glucose (glucose : maltose = 1·95). This experiment has given almost identical results to those with 5 g. of the unsterilised barley in Exp. 6, Table III (glucose : maltose = 1·93). In both cases practically the same proportion of the starch of the grain has been digested: in Exp. 6 the sugars calculated as starch represent 20·16 % and in Exp. 21, 20·5 % of the barley taken. This does not represent the total starch of the grain as shown by the results in Table VII, but only the amount digested in 48 hours. In the above calculation no allowance has been made for the pre-existent sugars in the germinated barley.

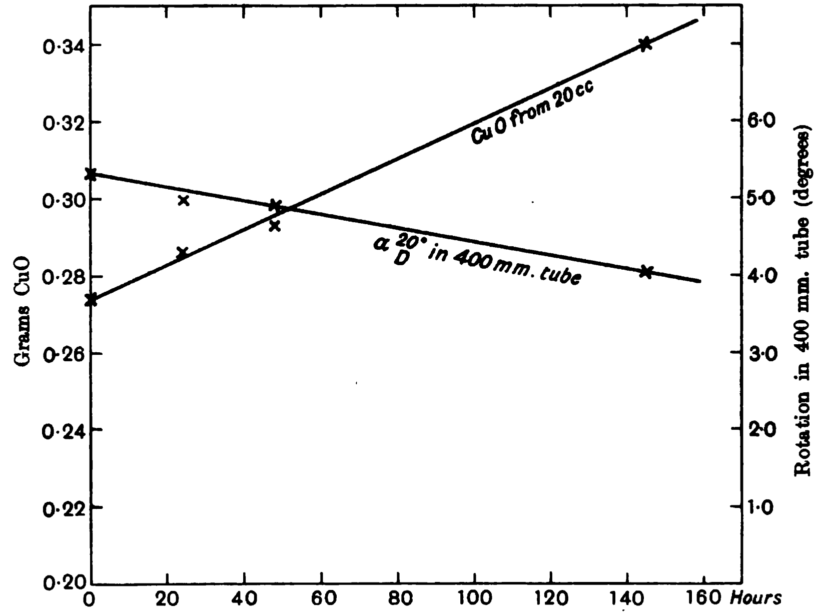

Fig. 1. Action of 0·2 g. of Germinated Barley on Maltose at 38°. (Table XI.)

TABLE XII.

Self-digestion of 5 g. of Germinated Barley.

Expt.	Mean CuO from 50 cc. of solution *K*	CuO from 50 cc. *K* after fermentation with			Glucose in 500 cc. *K*
		S. marxianus	*S. exiguus*	Mean	
21	0·2388	0·0526	0·0508	0·0517	0·7395

Expt.	Maltose in 500 cc. *K*	Glucose / Maltose	Starch in the form of sugars	Percentage of starch calc. on barley
21	0·3794	1·95	1·0252	20·5

SUMMARY.

1. It is shown that air-dried germinated barley contains a *maltase* which hydrolyses maltose to glucose. Attempts to separate this enzyme by extraction with water followed by precipitation with alcohol caused its destruction; the preparation obtained only acted on starch in the same way as ordinary malt extract, forming maltose and dextrin.

2. The presence of maltase in germinated barley is shown, however, by allowing the finely powdered grain to act upon starch or maltose at 38°. Under these conditions a large amount of glucose is formed in both cases; as the proportion of glucose increases, that of the maltose falls. There is no doubt that in the case of the starch the glucose arises wholly from preformed maltose and not directly from starch. The action on starch is very similar to that of taka-diastase, which contains maltase in addition to the ordinary diastatic enzymes.

3. When the germinated barley is allowed to digest itself for a prolonged period, practically the whole of the starch disappears and is converted into glucose. In the earlier stages of the action dextrin and maltose are found but they gradually disappear until only glucose remains. The action probably takes place in the series of stages:

starch ⟶ soluble starch ⟶ dextrins ⟶ maltose ⟶ glucose.

4. When a small quantity of germinated barley (0·2 g.) is used to digest gelatinised starch or maltose, the self-digestion of the barley starch is largely inhibited until the greater part of the added starch or maltose has been converted into glucose. This makes it impossible to apply a "correction" for the enzymic material by carrying out a control with the latter in presence of water only.

5. When the germinated barley (0·2 g.) digests gelatinised starch, dextrin, maltose and glucose are found even after very prolonged periods. The dextrin and maltose gradually and continuously fall, but even after 263 hours both are still present; the glucose steadily increases in amount during the whole period of digestion.

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VI. OBSERVATIONS UPON THE GROWTH OF YOUNG CHICKENS UNDER LABORATORY CONDITIONS.

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There are not many published records of the use of young chickens as the experimental animals in studies upon the factors concerned in growth and nutrition, but, from a reference to those of recent date, the present author was led to believe that one might rely to some considerable extent upon their suitability for such studies.

When the present work was begun, it was intended to make a full study of the effect of various diets upon tumour growth in the young, growing chick.

The particular tumour which was selected for use was one of the avian neoplasms described by Rous [1910]. This growth, originally found by him in a Plymouth-rock hen, simulates in many respects a spindle-celled sarcoma. It is very suitable for the study of the effect of diet upon tumour growth, on account of its high degree of virulence, its rapid growth, and its great tendency to form metastatic deposits in other parts of the body.

Peculiar interest is also attached to the growth from the fact that it may be transmitted by means of the cell free filtrate from the crushed tumour. For this reason, as well as on other grounds, many workers consider that the growth should be classified as one of the infective granulomata, rather than as one of the true sarcomata.

Whether this is so or not, is a matter which remains as yet undecided. Meanwhile it was felt that the tumour sufficiently simulated a true sarcoma to warrant an investigation along the lines previously mentioned.

At the outset of the work, Dr A. Paine, the Director of the Research Institute, brought to my notice an observation that he had made. He had been struck by the poor standard of development and nutrition reached by large batches of young chickens reared in the Laboratory animal room from an early age, and, in view of some of the published results from this Laboratory, it was felt that the matter was of sufficient importance to justify an investigation.

It will be useful if reference is made here to some of the earlier research which has been done upon the growth of young chickens and upon the effect of various dietaries upon the growth of Rous' sarcoma grafts which they carried.

Funk [1913] utilised, for a research of this nature, young chicks of the Plymouth-rock strain, which were a fortnight old when observation was begun upon them. They were divided into different batches, which were fed upon different variations of a rice diet. After the elapse of 15 days, each bird received an injection of Rous' sarcoma, and the effect of the diet upon the subsequent development of the tumour graft was then noted.

He found that the young birds, which received as their sole food cooked polished rice, invariably died after some fourteen days without exhibiting any apparent symptoms of beri-beri.

A further series of birds were fed entirely upon cooked "red" (unpolished) rice, and results of apparent importance were obtained upon this batch.

It was found that the chicks remained alive for some four weeks or so without manifesting any sign of further growth. During this period, Funk's control birds, fed upon chicken food, doubled their initial weight.

There is no need to deal here with the results of the dieting upon the injections of tumour material. They are strictly in accordance with the well-known fact that tumour grafts will not flourish well in an ill-nourished host.

Funk apparently kept observation upon his young chicks for a period of only twenty-eight days, a period, however, that he considered sufficiently long for his purpose, in spite of the fact that he realised to some extent the difficulty of rearing the little birds upon such monotonous diets. It is noteworthy that Funk did not observe any appreciable diminution of the appetites of the experimental animals.

In a later publication Funk [1914] corroborates his previous finding, that it is possible to arrest the growth of young chickens by feeding them upon a diet of "red" rice. A photograph is contained in his paper which contrasts very plainly the great difference in size and development of two chickens, one of which had been fed entirely upon a diet of "red" rice, while the other had received normal food.

Subsequently, in collaboration with Macallum, Funk [1914] published the description of extended studies upon the arrest of growth in young chicks. By adding a small ration of cod-liver oil to the diet of "red" rice, Funk and Macallum claim to be able to arrest the growth of young chicks over a much greater length of time than had been previously done upon a diet of "red" rice alone. They give somewhat detailed particulars of an individual bird which was maintained at an almost constant body-weight for a period of no less than five months by this means.

There is also appended to their communication another apparently striking photograph of this particular chick, standing by the side of a normal bird of the same age. It is not, however, stated whether the normal bird, whose photograph is reproduced, was reared in the Laboratory, and from the same series of chicks as the stunted one. In view of the results which will be shown in the present paper this question is one of the greatest importance.

The stunted chick possessed several peculiarities which were noted by Funk and Macallum. They found that the bird failed to develop any of the secondary sexual characteristics of the fowl, its voice is described as that of a four-week-old chick, and apparently the growth and development of the beak and feathers were not retarded to such a degree as was that of the body itself.

The present writer was so much impressed with the importance of the possibility of being able to stunt at will, for comparatively long periods of time, the growth of the young chick, that the preliminary experiments were directed to corroborate this finding, if possible.

Accordingly, in the early summer months of 1915, a large batch of young Plymouth-rock chicks was obtained from a reliable breeding farm in the country. The young birds arrived at the Institute at the age of ten days, and they were at once placed in a heated foster-mother where they were reared until somewhat older and stronger.

This was done as several preliminary experiments had shown that very young chicks are too delicate to be fed with unnatural dietaries.

The birds were strong and healthy when brought in, and, during the whole of the time that they were being hardened off in the foster-mother, they ate their food well. They were weighed regularly, and observation both of these weights and of their general appearance warranted the assumption that they would develop into healthy vigorous chickens.

It might be mentioned here that the foster-mother was constructed of wood, and the heating regulated so that as time elapsed the young birds might be gradually hardened off. The floor was covered with a layer of fine sand and grit.

The diet supplied to the birds during this period was mainly a special chick-food, chopped egg and bread crumbs, and fresh green food.

After having been in the foster-mother for a month, the birds were divided into four batches, each of which was placed in a separate pen. The pens were large and well ventilated, provided ample room for exercise, and possessed a covered-in end, fitted with a warm sleeping shelter. The floors of the pens were covered with coarse sand, shell mixture and sawdust. The animal-room, in which the pens were kept, is large and airy, and has been constructed upon scientific principles for the housing of experimental animals under as natural conditions as possible.

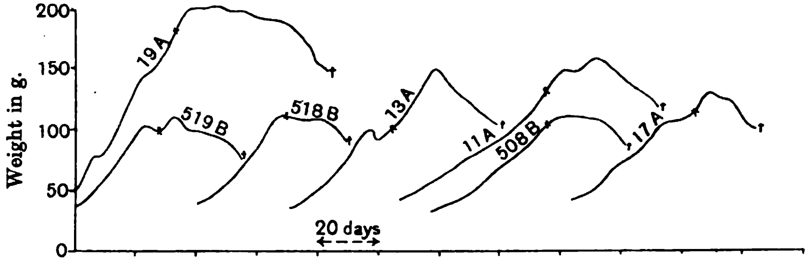

Chart I. Growth curves of chicks fed upon a diet of cooked "black" rice. × marks commencement of diet.

At this point in the investigation all the young birds were in good health as far as could be judged. They continued to eat well and the weekly increment of body-weight was satisfactory. All four batches were fed normally in the pens for a fortnight, receiving chicken-food, corn, meal and fresh green vegetables.

It was towards the end of this fortnight that the first cases of indisposition occurred. One or two of the chicks showed signs of weakness, particularly in the legs, and in these cases it was noted that the increase in body-weight was not as satisfactory as it had been.

Particular notice was not, however, paid to these birds, as it was considered that they might be exceptional cases.

They were accordingly weeded out, and the remaining chicks were placed upon various diets. One group received as sole source of nutriment cooked polished rice, a second was fed entirely upon cooked "red" (unpolished) rice, a third batch was given another variety of unpolished rice, namely, "black" rice, in the cooked state, whilst the fourth and last group was maintained on normal food. Daily observation was made on the birds, and they were weighed at intervals of four days. The birds which were fed upon "white" polished rice behaved in the manner that was expected. They almost immediately failed to register an increase in body-weight, then followed a period of rapid decline and finally death (Chart II). Failure of appetite did not seem apparent during the decline, until in the latter stages. There is little need to comment upon the results obtained in this series of birds, for the results are but a corroboration of those which have been published by a considerable number of workers.

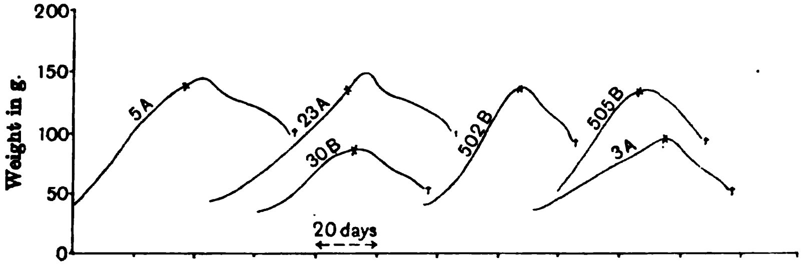

Chart II. Growth curves of chicks fed upon diet of cooked "white" rice.
× marks commencement of diet.

The second group of birds, which were given "red" rice alone, were watched with particular interest in view of the findings of Funk which have been referred to.

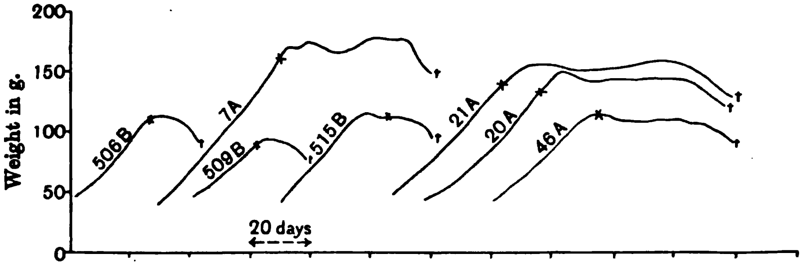

Chart III. Growth curves of chicks fed upon diet of cooked "red" rice.
× marks commencement of diet.

It was found, on an examination of the body-weights of the chicks in this batch, that growth was in many cases suspended shortly after the commencement of the feeding. Many of the birds did maintain an almost constant body-weight for periods of time varying from twenty to eighty days (Chart III). It was, however, soon obvious that the chicks were far from being in a healthy condition. The observations of Funk and Macallum with regard to the absence of any development of the secondary sexual characteristics and of the voice, and the comparative overgrowth of beak and feathers of these chicks, were confirmed. Many of them showed a weakness in the legs and they all presented a ruffled appearance. Again, as in the case of the animals fed upon "white" rice, no apparent failure of the appetite was observed until a few days before death, which usually occurred after a rapid decline, following the period during which maintenance was marked.

Post-mortem examination of the chicks which died in this batch failed to reveal many apparent abnormalities. The birds were emaciated and there was a poverty of blood in the organs, the majority of which were atrophic; particularly was this the case with the thymus and the spleen.

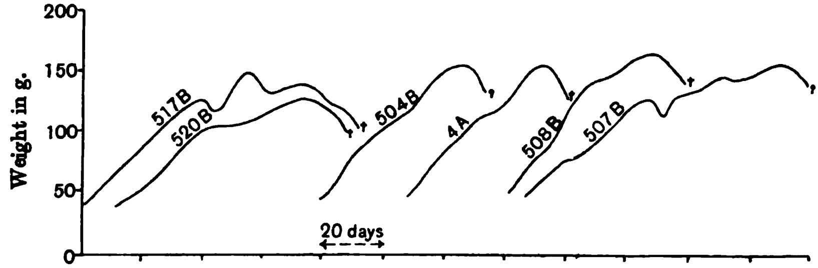

Chart IV. Growth curves of control chicks, showing early failure.

The third series of chicks, fed upon "black" rice, reacted to the diet in a very similar manner to those which had received "red" rice. This is a matter for no surprise when one considers that the two diets are very similar. If anything, the period during which growth was stunted was shorter, and the final decline more rapid than in the case of the chicks fed upon "red" rice (Chart I).

The really interesting results of the work, however, followed, strangely enough, from a study of the remaining batch of chicks which had been retained upon normal diet, to act as control animals to the other three groups (Chart IV).

Mention has already been made of the one or two cases of weakness which were observed before the chicks were placed upon special diets.

It was now found that further similar cases occurred with increasing frequency amongst the control birds. Taken collectively, the batch began to present the same ruffled appearance and weakness in the legs as those which were fed solely upon rice diets, while few of them continued to put on weight.

Only one particularly strong chick continued to grow at a rate at all approximating to normal. There were, on the other hand, several cases of arrest of growth over considerable periods of time, followed occasionally by a slow resumption of a very subnormal rate of growth, or, as was the case with the majority, by a rapid decline and death (Pl. I, photo. 2). Few of these birds lived longer than 120 days, which is little better than those which had been fed solely upon "red" rice.

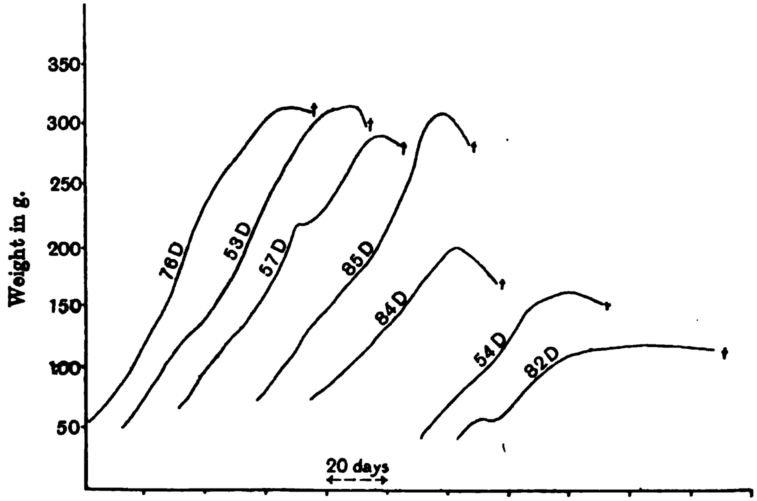

Chart V. Curves showing failure of young chicks reared upon earth surface.

Even the one bird referred to above did not maintain its superior rate of growth for long. After fourteen weeks it arrived at its maximum body-weight of 500 g. and it was then a fairly strong chick (Pl. I, photo. 1). Subsequently it maintained its body-weight for some few weeks after which it declined in the same way as had done the other birds of this group.

The results obtained in the series of experiments may be tabulated in the following form:

No. of chicks in batch	Diet	Average length of life, days	Average maximum body-weight attained, g.	Average weight at death, g.
12	normal	123	256	216
12	"white" rice	65	107	91
12	"red" rice	82	167	100
12	"black" rice	76	162	113

From these average results it will be seen that the consequences of feeding "red" rice to young chicks is not much more detrimental to the nutrition of the animal than if one retains the bird on normal food.

The failure of the normal birds to grow and develop was at first attributed to possible defects in the condition of keeping or feeding. Thus, poultry-keepers sometimes consider that weakness in the legs is due to a deficiency of fresh meat in the diet. It was therefore attempted to rear a batch of young chicks upon normal food, giving them in addition a ration of fresh meat daily. The birds ate the meat greedily, but did not respond to the change, and the effort to raise them was no more successful than before.

It was then thought that if the young birds were reared upon a more natural surface they might thrive more satisfactorily. Accordingly, two batches were reared separately, one being brought up upon a surface of sawdust and coarse sand, scattered on the floor of the cage (Chart VI); whilst the other group was provided with a surface of fresh earth and grass, which was frequently changed (Chart V).

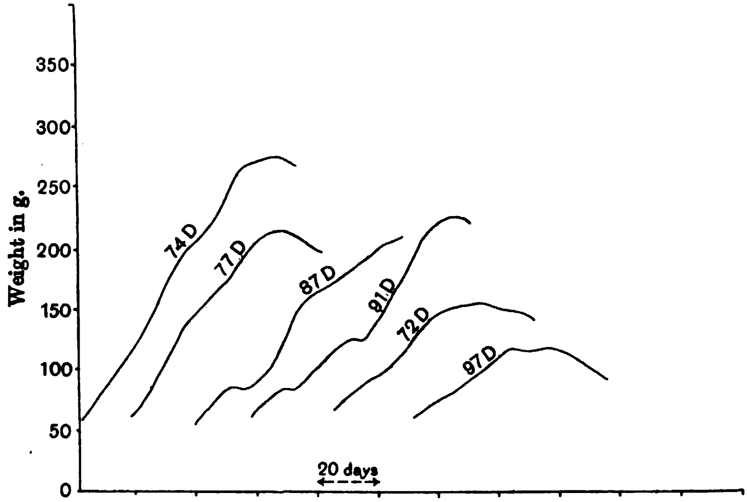

Chart VI. Curves showing failure of young chicks reared on a surface of sawdust and sand.

Observation upon both batches was made as before, but very little improvement was noted in those reared under the more natural conditions. Certainly, as is seen from the tabulated results, they lived on the average somewhat longer, and attained a slightly greater maximum body-weight, but the usual symptoms of weakness, which have been described, made themselves apparent sooner or later and the chicks declined and died.

No. of chicks in batch	Reared upon	Average length of life, days	Average maximum body-weight attained, g.	Average weight at death, g.
12	Sand and sawdust	80	173	168
12	Earth and grass	93	239	229

Post-mortem examination of a large number of these "normal" chicks did not bring to my notice any particular point of interest. The bodies were without exception greatly emaciated. The organs were usually small and anaemic. During life this apparent poverty in blood was striking. The combs and eyelids of the chicks were peculiarly bloodless, being of a dead white colour, as contrasted with the deep red colour possessed by the combs and eyelids of normal fowls of an equal age.

The results were of so striking a nature, and of such great importance in a consideration of the value that should be placed upon results of previous investigations carried out upon young chickens, that the experiments which have been described were repeated upon a further batch of young Plymouth-rock chicks.

This series were hatched out from eggs in the Institute, and were thus under observation from their birth. They were a strong healthy brood when born, and they were carefully reared in the foster-mother until five weeks old. They were then divided into similar batches to the other series, placed out in pens, and fed upon similar dietaries.

The results obtained were identical in every way with those which have already been described. The onset of the symptoms of weakness was, however, earlier in every case, and consequently the birds did not live so long or attain so great a weight.

No. of chicks in batch	Diet	Reared upon	Average length of life, days	Average maximum weight attained, g.	Average weight at death, g.
10	normal	earth	81	202	171
10	normal	sand	74	187	165
10	"red" rice	,,	45	103	87
10	"white" rice	,,	41	108	80
10	"black" rice	,,	47	112	80

There now seemed every reason to believe that the observation communicated to me by Dr Paine was substantiated by facts, and that it was indeed a matter of difficulty to rear young chickens at all under the artificial conditions of an indoor animal-room.

While this work had been in progress, it was observed that a batch of Plymouth-rock hens which had been brought into the Institute at the age of six months were continuing to grow at a normal rate and appeared in every way healthy and strong. This observation suggested that an investigation into the effect of the age at which the young chickens were brought into the unnatural surroundings upon their subsequent development might yield useful data, particularly as large numbers of full-grown birds have been kept for long periods of time, under the same unnatural surroundings, without in any way showing any tendency to decline. Even egg production occurs in these full-grown fowls at a normal rate.

For the purpose of this research a large number of young Plymouth-rock chicks were hatched out in the country and were then brought into the Institute after various periods of time. Observation was kept on them closely after they had been brought into the artificial conditions and the results have been tabulated as follows (Chart VII):

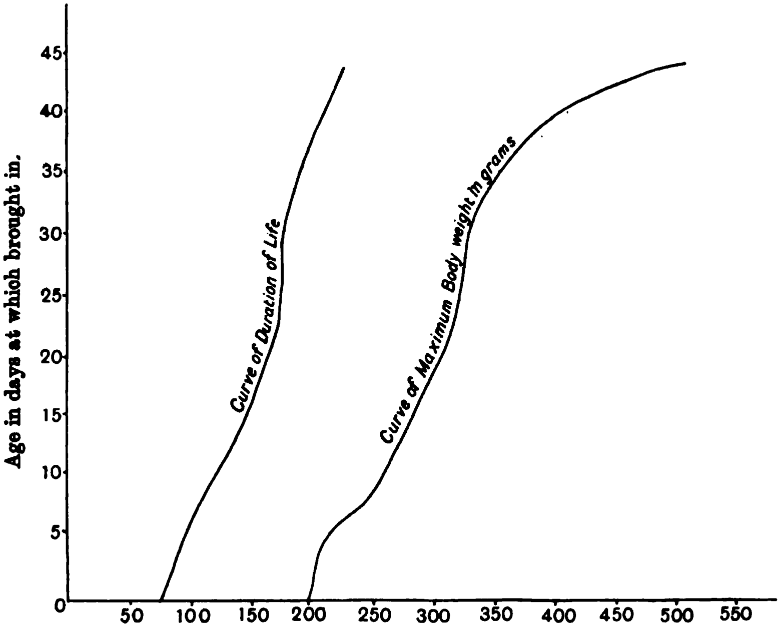

Chart VII. Body-weights in g. Duration of life in days.

No. of chicks in batch	Age brought in, days	Average length of life, days	Average maximum body-weight, g.	Average weight at death, g.	Remarks
20	0	77	197	168	All died exhibiting signs of extreme malnutrition
16	4	86	206	198	ditto
25	7	84	233	184	ditto
12	10	123	256	216	ditto
12	21	152	307	290	Symptoms not so severe, yet all died from malnutrition
8	28	160	320	300	ditto
10	42	6 yet alive after 205 days	497	—	Poorly nourished and ill developed. Six yet alive, but all declining rapidly

The ten birds in the last group have resisted the unfavourable influences of the unnatural surroundings longer than any other batch have done. They have never presented, even at the time when they registered a maximum body-weight, a strong appearance.

During the period of decline they have not shown the symptoms of weakness to anything like the same extent as did the first four batches, nor did they possess the pallor of the comb and eyelids which was so characteristic of the earlier groups of birds.

We now come to a consideration of the results which have been obtained. Firstly, it is felt that the young chick is not a suitable subject upon which to conduct experiments in the study of growth, at any rate, when it is kept under artificial conditions. The difficulty of rearing young fowls indoors is, I believe, realised by poultry-farmers generally, so much so in fact, that it is seldom attempted by them.

An explanation of the cause of the failure has not yet suggested itself. No dietary deficiency can be held as responsible, for the diet supplied to the experimental chicks in the normal batches was in every way similar to that successfully employed to rear chicks in the open air. An attempt to supply one of the conditions, under which fowls are normally reared, was made when fresh earth and grass were given to provide a surface for the young birds to run upon.

They certainly made use of the soil, scratching it over and picking out small insects and other tasty morsels all day long. And yet, despite the fact that the earth was changed frequently, this group of birds reached but a slightly better standard of development than did a similar batch, which had a surface of sawdust and grit to run upon.

In the light of these facts we must admit that there are other factors than an adequate diet, which must be supplied to the growing chick, in order that it may complete its normal growth when under conditions such as are described.

If we submit the results which have been published by Funk, and by Funk and Macallum, upon growth in young chicks, to a criticism directed along these lines, we are forced to conclude that they now have a much smaller value.

It is unjustifiable to deduce results from the feeding of an experimental animal upon a certain diet, unless the control animals, which are fed normally, behave in a normal manner. In the present case, it has been shown that young chicks may be fed upon a normal dietary—which under better surroundings would suffice to produce a normal development—and yet suffer from severe forms of malnutrition.

We know well that there was no reason to expect the batches fed upon cooked "white" rice to behave in any manner other than they did, for it is definitely established that "white" rice is an inadequate diet in many respects.

It is however considered that it is unwise to draw any conclusions from the results obtained in dieting birds upon "red" rice. Although it is true that many of the birds which were fed in this manner, in this series of researches, did show a period during which arrested growth was a prominent feature; yet, many similar cases have been observed in the control chicks fed on normal food.

The photographs shown in this communication demonstrate that arrested growth may occur in normally fed chicks to almost as striking a degree as in the case recorded by Funk and Macallum.

The previous work of the authors just referred to, on growth in young chicks, therefore affords no satisfactory evidence of the existence of any accessory substances or, as Funk prefers to call them, "vitamines."

Needless to say, no attempt was made to carry out the proposed scheme of investigation into the effect of diet upon tumour growth in the young growing chick, nor will such an attempt be made in this Institute until it has been found possible to rear young birds indoors to a satisfactory standard of nutrition.

The observations reported in the present communication may, at first sight, seem incomplete without containing a more exhaustive enquiry into the cause which underlies the early failure of the young chickens. The advisability of carrying out such an investigation was, however, fully considered at the time, but it was felt that such a study would lead too far away from those problems which are, at the present time, engaging the activities of this Institute.

It is for this reason, therefore, that the results that were obtained have been communicated in their somewhat incomplete form.

SUMMARY.

1. It has been found impossible to rear, to a satisfactory degree of development, young chicks kept under the artificial conditions prevailing in this Laboratory.

2. The age at which the chick is brought under the influence of these artificial conditions is apparently the chief determining factor in its subsequent development, other conditions being the same.

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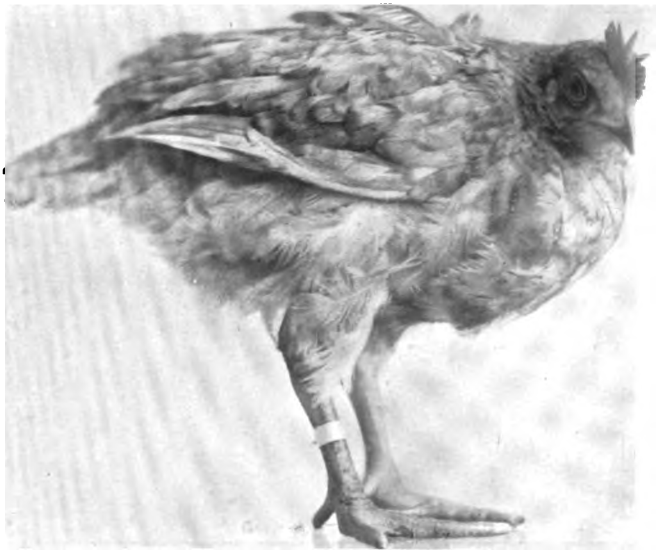
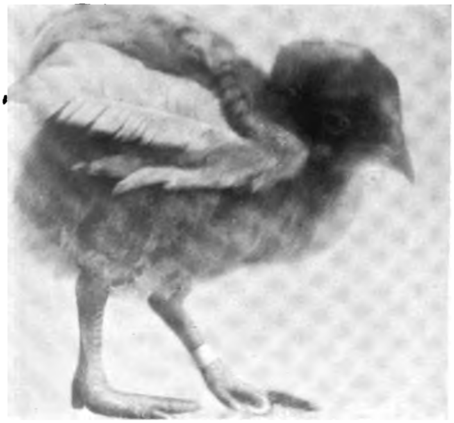


Photograph 1. *Chick* 1 *A*, fed upon normal food, age 14 weeks, weight 500 g. At the time that this photograph was taken, this bird was at its prime. Nevertheless, the comb and eyelids were very pale and the bird was not strong. This chick remained at a body-weight of about 500 g. for nine weeks before it declined and died.



Photograph 2. *Chick* 207 *A*, age 12 weeks, weight 147 g. This bird was also fed upon normal food and belonged to the same batch as No. 1 *A*. It fed well, and seemed quite as strong as the bigger bird, despite its ill-developed appearance. The overgrowth of the wing feathers may be noted in this case. This chick remained at an almost constant body-weight for 80 days before it declined and died. Both photographs are on same scale.

VII. THE GROWTH OF RATS UPON ARTIFICIAL DIETS CONTAINING LACTOSE.

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(*Received December 24th, 1915.*)

It has been known for some time that many natural food-stuffs contain substances which, although in many cases present in very small quantities, play an exceedingly important rôle in the processes of growth and nutrition.

A large amount of literature has been published during recent years which deals with these substances in one way or another. Particularly has interest been centred upon those substances of this class which if absent or deficient from the diet may induce a definitely pathological condition.

We are, however, in this paper concerned with another group of these so-called accessory substances, namely, those which are apparently indispensable to the diet of a growing animal if satisfactory growth is to occur.

Milk has been shown to be peculiarly rich in its content of growth-promoting accessory substances. Hopkins [1912] showed that a very small ration of milk added to a diet consisting of purified caseinogen, starch, lard and salts—a diet that by itself was inadequate to supply all the factors necessary for the growth of young rats—at once rendered it sufficient for this purpose. Stepp [1909, 1911, 1913] had previously shown that dietaries which had been extracted with alcohol were rendered incapable of sustaining the growth processes of young mice, but when the extracted substances were completely replaced normal growth was at once possible.

Stepp demonstrated that the replacement of many of the individual components of the alcoholic extracts, such as purified natural fats and lipoids, was not followed by any improvement in the animal's condition.

He therefore assumed that the factors responsible were in all probability some unknown members of the class of lipoid substances.

Feeding experiments were accordingly made by several workers, in which attempts were made to rear young growing rats to maturity upon fat-free diets.

Osborne and Mendel [1912] carried out an extensive series of experiments of this nature, using diets similar in composition to those utilised by them in their earlier researches [1911], but which had been subjected to extraction with ether. With such diets they found that it was possible to obtain satisfactory growth in young rats, over a period of time equal to that during which the rat normally shows growth, and concluded that growth is possible upon a fat-free diet.

Since their diets had not been extracted with hot alcohol, they naturally could not claim that they were lipoid-free, and they were even forced to admit that they had not been able to obtain growth in young mice when fed upon diets similar to those employed by Stepp. In spite of these facts, however, the views of the latter author were subjected to criticism.

That the active substance or substances may in some cases be removed from diets by treatment with fat solvents was shown when McCollum and Davis [1913] announced that an ether extract of butter added to a diet, inadequate in accessory substances, at once rendered the diet satisfactory. Osborne and Mendel [1913, 1, 2] also demonstrated that unsalted butter and butter fat possessed the same power.

It was then soon found that many naturally occurring oils and fats have this property associated with them, while, on the other hand, many other representatives of this class are devoid of any such activity.

McCollum and Davis, in the communication referred to above, also attempted to feed young rats upon a fat-free diet, composed of caseinogen, lactose, agar, dextrin and a suitable salt mixture. They too recorded results which apparently demonstrated that growth for a certain period of time was possible upon such a diet, but that, invariably, sooner or later, the animals fed upon it declined and died. This decline and death was not the result if the diet contained the ether extract of butter or egg yolk, for then the animals grew normally in every way.

While much work was being done upon the presence of the accessory substances occurring in company with many of the fats and oils, the possibility of other fractions of a normal dietary also containing other unknown substances possessing equally important properties was almost neglected.

Funk [1914, 1] and Funk and Macallum [1914] have, however, subjected Osborne and Mendel to criticism for the extensive use which the latter authors make of "protein-free milk." In this criticism it was pointed out that it was unsatisfactory to introduce into diets such an unknown factor as this complex mixture represents. McCollum and Davis [1915, 1] have also criticised the use of "protein-free milk," and have pointed out that the very differences, recorded by Osborne and Mendel themselves, in the nutritive value of natural "protein-free milk," as compared with an artificial product made from lactose and salts, were sufficient to render evident the existence of substances of an unknown nature, other than those usually carried by certain fats.

In this respect an observation made by Stepp is of interest. He favoured the belief that the substances present in milk, which play the part of accessory substances, are present not in the fat but rather in the skimmed milk fraction.

That there was any serious objection to the use of lactose in diets employed in feeding experiments has, however, not been considered fully until recently, when Hopkins and Neville [1913] repeated some of the earlier work of Osborne and Mendel. The former authors found that when purification of the various ingredients of an artificial dietary was carried to a further degree than usual, the animals declined at a much more rapid rate than when fed upon a similar diet which was less pure. In the preparation of the purified diets, they purified the caseinogen and starch by extraction with alcohol, and several times reprecipitated the lactose from aqueous solution by alcohol. Their results demonstrated clearly the importance of purifying the lactose employed in dietaries. Funk [1914, 1] also speaks of commercial lactose containing traces of nitrogen and therefore considers it is an unreliable ingredient to include in diets which are to be considered as "pure."

McCollum and Davis [1915, 1] have remarked that they appreciated the possible objection to the use of insufficiently pure lactose in diets. The samples of this sugar used by them, in their series of studies upon growth on fat-free foods, contained amounts of nitrogen varying from 0·02–0·034 %. In view of this fact they wisely refrained from any discussion as to the necessity of "vitamines," or hormones in the diets, until they had made an investigation of this point.

Sweet, Corson-White and Saxon [1915], in a research upon the influence of diet upon tumour growth in the rat, used diets similar to those employed by Osborne and Mendel, and they had occasion, from a consideration of their results, to believe that lactose does in some cases carry with it impurities which may act as accessory substances to promote growth. They even explain some results obtained previously by van Alstyne and Beebe [1914], working upon the same subject, upon these grounds. The latter workers had fed rats upon two similar purified diets, one of which contained lactose, while the other did not. Investigations were made upon the effects of these two diets upon the growth of tumour grafts inoculated into the rats. From the results it was concluded that the carbohydrate of the diet, i.e. lactose, had had a beneficial effect upon the growth of the inoculated tumours in the rats. Sweet, Corson-White and Saxon now suggest that this result may be more correctly attributed to the presence of a growth-promoting accessory substance in the insufficiently pure lactose used in the diets.

In a recent paper Woglom [1915] has investigated the effect of lactose in diets upon the growth of tumours in rats and mice. The lactose was, however, added to their normal diet of bread and vegetables. From his results he concludes that lactose does not increase the receptivity of mice and rats for the transplantable carcinomata of their species and he proceeds to criticise the previous work of van Alstyne and Beebe. It must be remembered, however, that the latter authors added their lactose to a carbohydrate-free diet of caseinogen and lard, an important fact which Woglom has apparently passed over. The explanation of the results of van Alstyne and Beebe which has been given by Sweet, Corson-White and Saxon is, in all probability, the correct one. Any improvement in tumour growth in animals fed upon dietaries containing lactose is due, not to the lactose itself, but rather to the growth-promoting accessory substance which it so often carries, when insufficiently purified.

This work of Woglom therefore loses much of its value from the fact that he added the lactose to a diet already fully sufficient for normal growth. Consequently any action of the accessory substance, carried by the lactose he used, would fail to be apparent since both host and tumour-graft were already able to derive all the substances necessary for growth from the basal diet, without any addition of lactose.

Since the early spring of 1915, I have been making a large number of feeding experiments upon rats, with the object of obtaining sufficient experience in the technique to permit me to make an extensive examination of the effect of diet upon tumour growth. It was during these preliminary feeding trials that I was struck by the apparent effect of the purity of the lactose used upon the growth of the animal. Accordingly I made a few experiments to ascertain the cause of this effect, and the results I have obtained are set forth in the present communication.

While the results were being compiled for publication, however, three papers appeared by McCollum and Davis [1915, 2, 3, 4], in which these indefatigable workers also record results consequent on the use of impure lactose in dietaries. They there put forward sufficient evidence to show that lactose, unless carefully purified, often does contain an accessory substance which helps to promote growth.

The identical conclusion to which the writer of the present paper had arrived, as a result of independent experiments, will now serve as a useful corroboration of the work of McCollum and Davis.

In view of the observations of Stepp, Hopkins, Funk and earlier workers, it has seemed a difficult matter to explain satisfactorily how Osborne and Mendel, and also McCollum and Davis, had been able to obtain a certain degree of growth in young rats fed upon fat-free diets composed of "purified" food-stuffs.

Osborne and Mendel have always been careful to utilise, throughout their extensive researches, proteins of a very high degree of purity, and yet they have gone to the other extreme in continuing to use in their diets "protein-free milk."

Reference has already been made to the criticisms which have been directed against the use of this complex substance, as a source of lactose and salts.

To determine whether any substance was contained in "protein-free milk" which would exert a growth promoting influence, two batches of young rats were taken and were fed upon the following diets.

Diet I.		*Diet II.*	
Caseinogen	18·0	Caseinogen	18·0
Wheaten starch	30·0	Wheaten starch	30·0
Lard	22·0	Lard	22·0
"Protein-free milk" ...	30·0	Extracted "protein-free milk"	30·0

All the ingredients of the diet other than the "protein-free milk" were carefully purified. The caseinogen was precipitated from machine-skimmed milk by acidification. It was then reprecipitated twice from dilute solution in sodium carbonate by the addition of acid, washed, pressed and dried.

The dried caseinogen was extracted with two changes of alcohol for six hours at 60°, and finally subjected to ether extraction for six hours.

McCollum and Davis [1915, 4] have now shown that caseinogen should be purified very carefully, owing to the fact that changes may occur in the protein molecule, if it is subjected to purification by strong measures, which may greatly lessen its nutritive value.

I have not found that the purification, to which I subjected the caseinogen used throughout these experiments, has rendered it unsuitable for the rats to any marked degree. The only two occasions on which I suspected that the caseinogen was unsatisfactory for the nutritive demands of the animals were when it had been extracted with hot alcohol whilst in a slightly moist condition. In these cases every rat upon the diets containing this caseinogen died within a short space of time. I have not yet investigated the matter carefully, but the result may be attributable to the caseinogen having undergone changes of an unknown nature.

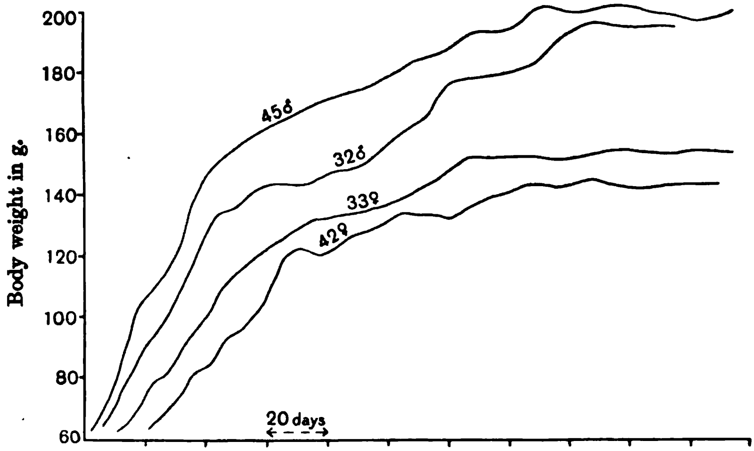


Chart I. Curve showing normal growth of young rats upon diet:

Milk powder	60
Lard	24
Starch	16

The wheat starch was also submitted to alcohol and ether extraction, while the lard was the purest obtainable.

The extracted "protein-free milk" was prepared by drying the product, obtained by following out the directions of Osborne and Mendel [1911], in an oven at 100° for six hours. The dried mass was then ground to a fine powder and extracted with alcohol at 60° C. and finally with ether.

To test whether the "protein-free milk" had been affected by the six hours' exposure to 100°, a third series of rats was placed upon a diet similar to Diet I, but which differed in containing the heated product.

Reference to Charts II, III, IV will show typical growth curves of rats fed upon these three diets, as compared with normal growth upon a milk powder diet, Chart I.

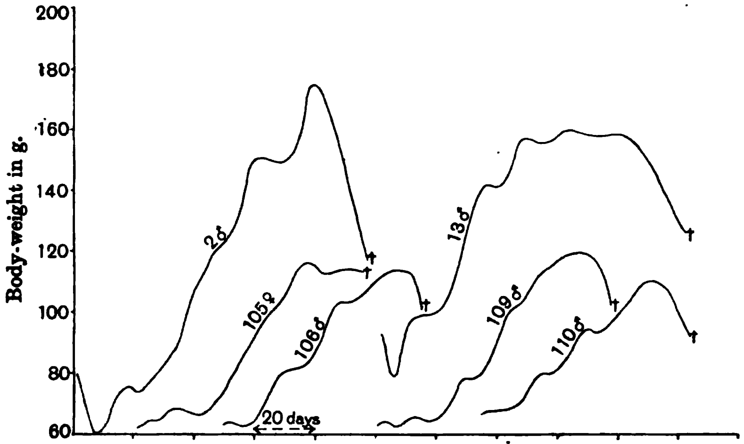


Chart II. Growth of young rats upon diet of:

Alcohol extracted caseinogen	18
Alcohol extracted starch	30
"Protein-free milk"	30
Lard	22

Growth was obtained upon the diet containing "protein-free milk" and also to about the same degree upon that containing the heated product. The growth curves in Chart II are in every respect similar to those published by Osborne and Mendel, using similar diets. Approximately normal growth occurs for periods of from sixty to eighty days, and is followed by a cessation of growth and a rapid decline. This cessation and rapid decline may be prevented, and the animal may continue to grow normally, if at the critical point one of the fats containing accessory substances is added to the diet.

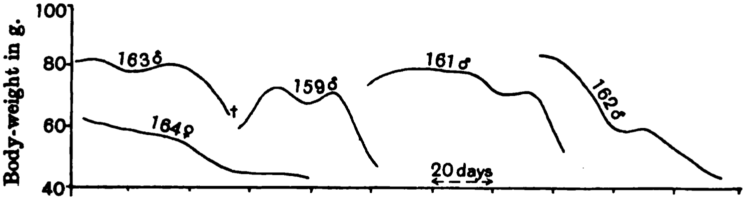


Chart III. Failure to grow of rats fed upon diet:

Alcohol extracted caseinogen	18·0
Alcohol extracted starch	30·0
Heated and alcohol extracted "protein-free milk" ...	30·0
Lard	22·0

The rats fed upon the diet containing heated and extracted "protein-free milk," however, showed no signs of growth whatever (Chart III). Slow decline characterised the period during which they were fed upon the diet, and this decline usually terminated in death after some forty days or so.

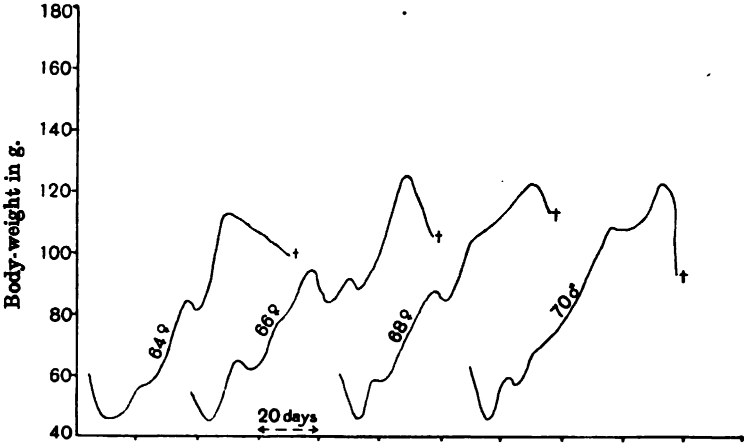


Chart IV. Growth upon diet:

Extracted caseinogen	18·0
Extracted starch	30·0
Lard	22·0
Heated "protein-free milk"	30·0

Obviously, some substances had been removed from the "protein-free milk," by the alcohol extraction, that were indispensable.

Similar results, I find, are recorded by MacArthur and Luckett [1915] who, investigating the indispensability of lipins in the diet, also subjected their "protein-free milk" to a similar extraction in order to remove from it all traces of this class of compound.

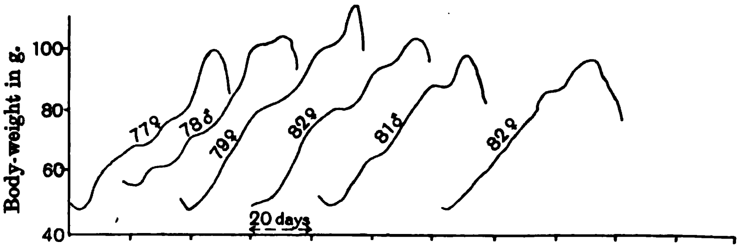


Chart V. Slow growth, and subsequent failure of rats fed upon diet:

Alcohol extracted caseinogen	18·0
Alcohol extracted dextrin	56·0
Alcohol extracted agar	2·3
Salt mixture *B*	3·7
Lactose	20·0

Experiments of a similar nature were then made, using a fat-free diet which has been used extensively by McCollum and Davis [1915, 1].

Diet IV.

Caseinogen	18·0
Dextrin	56·0
Lactose	20·0
Agar	2·3
Salt mixture	3·7

The salt mixture employed was one used with satisfactory results by these authors, and is composed of:

NaCl	0·173 g.
$MgSO_4$ (anhyd.)	0·266 „
$NaH_2PO_4H_2O$	0·347 „
K_2HPO_4	0·954 „
$CaH_4(PO_4)_2\ 2H_2O$	0·540 „
Calcium lactate	1·300 „
Ferric lactate	0·118 „

Iodine was supplied in the drinking water (distilled) once weekly.

Upon this diet it was found that the stronger of the rats grew at a satisfactory rate for a period of about 50 days (Chart V). They did not reach as high a maximum weight as did the series fed upon Diet I, i.e. a diet in which the lactose was in a cruder form,—and having grown as indicated, they soon rapidly declined and died. Again in this case, the decline and death could be prevented by the prompt addition to the diet of a fat-soluble accessory substance.

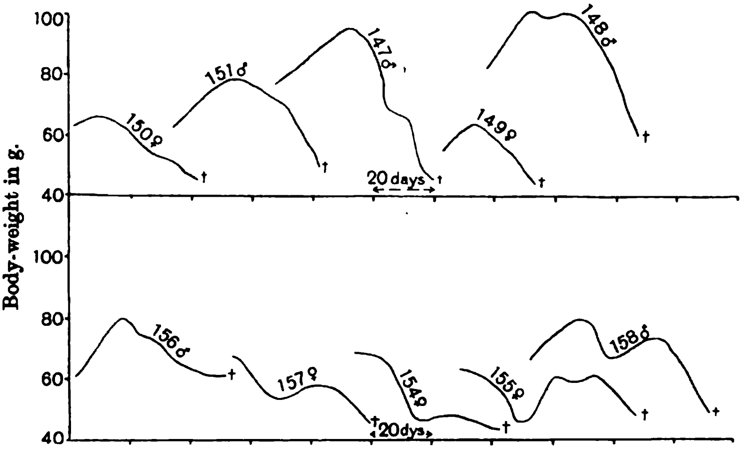
As all the ingredients of this diet, other than the lactose, had been subjected to careful purification, the results obtained showed that it would be advisable to investigate the effect of purifying the lactose.

The lactose used throughout these experiments was bought from reliable English firms as "pure," but notwithstanding this it usually gave, on analysis, evidence of the presence of nitrogen. The average figure obtained from analyses made on several samples of the sugar gave the percentage content of nitrogen as 0·024 %.

Samples of lactose as supplied under the description of "pure" and "purest" vary largely in their nitrogen content. I have had samples which were practically nitrogen free, and I can now attribute some of my failures to obtain growth upon such diets as Diet IV to this cause. The lactose was purified as Hopkins and Neville have described, namely, by precipitation from aqueous solution by means of alcohol.

A ration similar to Diet IV was then made up, containing the once recrystallised lactose instead of the cruder product.

The curves, showing the slight growth of rats fed upon this Diet V (Chart VI), demonstrate fully that the one purification of the lactose by recrystallisation had removed the majority of the growth promoting factor.



Charts VI and VII.

Chart VI. Slight growth, followed by rapid decline, upon diet:

Alcohol extracted caseinogen	18·0
Alcohol extracted starch	56·0
Alcohol extracted agar	2·3
Salt mixture B	3·7
Recrystallised lactose	20·0

Chart VII. Practically no growth upon diet:

Alcohol extracted caseinogen	18·0
Alcohol extracted starch	46·0
Alcohol extracted agar	2·3
Salt mixture B	3·7
Recrystallised lactose	20·0
Lard	10·0

A point of interest may be noted here. Using a diet similar to V, but containing some 10 % of lard, I was unable to obtain, in a large series of rats, more than one or two cases showing any growth whatever. The majority of the animals in this group slowly declined from the outset (Chart VII).

I cannot say, as yet, whether this result was purely accidental, or whether it had its origin in the inclusion of the lard in the diet. The possibility of the latter view being correct is not altogether out of the question, as parallel cases do exist. For instance, it is known that the quantity of carbohydrate in a diet plays a very important part in governing the utilisation of the accessory substance which prevents beri-beri [Funk, 1914, 2; Braddon and Cooper, 1914].

Another diet was then tried which contained a much larger percentage of the unpurified lactose.

Diet VI.

Caseinogen	18·0
Lactose	56·0
Dextrin	20·0
Agar	2·3
Salt mixture	3·7

It was expected, from the greater percentage of lactose in this diet, that growth would be still more successfully promoted, and experimentally this expectation was realised (Chart VIII).

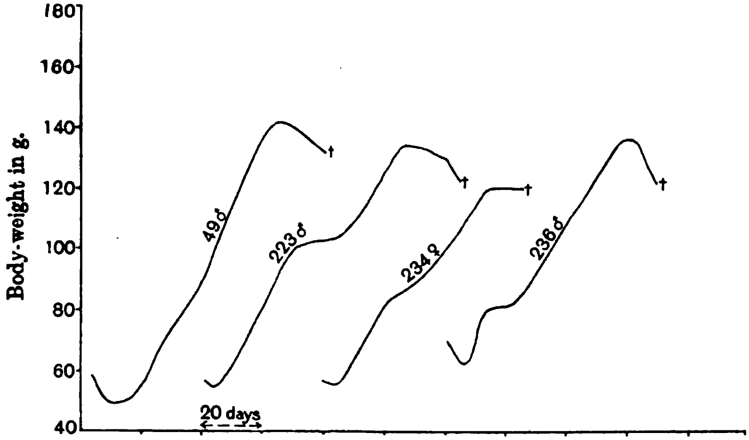


Chart VIII. Growth and early failure of young rats upon diet:

Extracted caseinogen	18·0
Extracted lactose	56·0
Extracted dextrin	20·0
Extracted agar	2·3
Salt mixture B	3·7

The animals survived, as a whole, longer, grew to a slightly greater maximum body-weight, and the subsequent decline did not occur at such an early date as in the cases of the rats fed upon a similar diet, which, however, contained only 20 % of lactose.

McCollum and Davis have just pointed out [1915, 3] that, if the lactose in a diet similar to this is entirely replaced by dextrin, no growth whatever is obtained, whilst Lander [1915] found no growth in young rats fed upon purified diets containing cane-sugar instead of lactose.

In another series of feeding experiments, lactose was used which had been twice recrystallised in the manner already indicated. No growth at all resulted, and the rats rapidly declined and died [Chart IX].

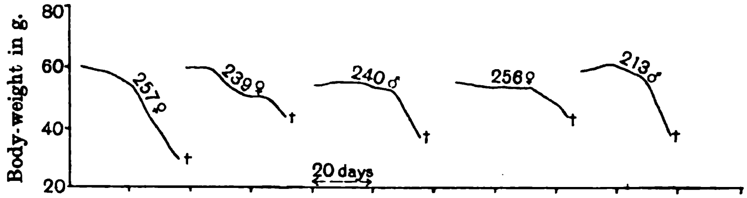


Chart IX. Rats fed upon diet:

Caseinogen	18·0
Dextrin	56·0
Agar	2·3
Salt mixture	3·7
Twice recrystallised lactose	20·0

A preliminary attempt was made to concentrate the active substance to some extent in the following manner.

The mother-liquors from a series of recrystallisations of lactose were concentrated down to dryness at 40° under diminished pressure. The resulting impure lactose was then recrystallised once from aqueous solution by means of alcohol. The mother-liquor from this recrystallisation was evaporated as before to dryness, and the product obtained was utilised in a diet, as the source of lactose, to the extent of 20 %. The effects of this concentration of the accessory substance upon the growth were not as marked as were expected. Chart X illustrates the type of growth curve of rats fed upon this ration. The maximum body-weight attained was slightly greater than that reached upon Diet IV, but no postponement of the rapid decline was observable. A further attempt is being made in this direction.

One cannot help being much impressed by the apparent need of the growing animal organism for more than one type of accessory substance. The one factor that has been studied in this paper is itself useless to make good the inadequacy of a pure diet so that complete growth can result. This can only occur when the diet also contains one of the fat-soluble accessory substances.

McCollum and Davis, in the recent papers referred to, have shown that many food-stuffs carry a water-soluble accessory factor that is of quite as great importance as those usually associated with many fats.

Thus wheat-embryo, egg-yolk and other substances are shown to contain water-soluble substances which may now be classified as accessory substances. Oseki [1914] had already shown that the accessory substance present in rye is soluble in water, while Funk and Macallum [1914] have suggested the likelihood that two classes of accessory substances—called by Funk "vitamines"—exist, one occurring in water-containing food-stuffs, such as fruit, whilst the other is generally found in the company of fats such as butter and cod-liver oil.

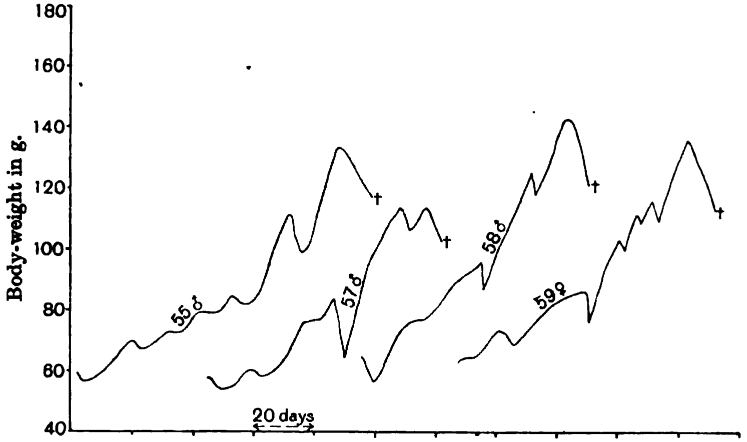


Chart X. Growth upon diet:

Caseinogen	18·0
Dextrin	56·0
Agar	2·3
Salt mixture B	3·7
Lactose + concentrated impurities	20·0

CONCLUSIONS.

It is apparent, from a consideration of the results set forth in the present communication, that lactose may contain effective amounts of a growth promoting factor, soluble in alcohol and stable on exposure to 100° for six hours.

These results are in entire agreement with those recently published by McCollum and Davis, and both sets of conclusions show that the suspicion with which other workers, to whom reference has been made, have regarded the inclusion of insufficiently pure lactose in experimental diets was well grounded.

It is now established conclusively that the views, held for some time past by Stepp, Hopkins, Funk and other workers, are correct, and that growth is impossible in the young animal when the diet supplied to it is deficient in certain indispensable accessory substances.

Our knowledge of the action of these unknown factors is so slight, that we shall be forced to reconsider many of the conclusions which have been drawn from the results of feeding experiments, involving the use of diets which we now know were not pure.

Much extremely valuable work has been done in this field, but the fact that many of the diets employed contained lactose, in an impure form, will make one await with interest the results which will be obtained when these experiments are repeated with diets containing a purer carbohydrate.

In this light, we may now regard many of the failures to obtain growth in young rats upon artificial food rations, which have been recorded by numerous workers, as evidence that they were truly dealing with diets of purified substances, and not as being due to faulty technique as has been suggested by some workers, whose own methods are now open to criticism.

SUMMARY.

1. Lactose, prepared from milk, may contain traces of an impurity which acts as a growth-promoting accessory substance.
2. This substance is soluble in water and alcohol, and is not destroyed by exposure to 100° for six hours.
3. Before complete growth can occur in a young animal, the diet, besides being adequate as regards its proteins, carbohydrates, fats and salts, must contain both fat-soluble and water-soluble accessory substances.
4. No growth is possible in the animals fed upon a purified diet which is entirely deficient in these accessory substances.

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VIII. NOTE ON THE ISOLATION OF METHYLGUANIDINE BY THE SILVER METHOD.

By ARTHUR JAMES EWINS.

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(*Received February 3rd, 1916.*)

In the course of some experiments which were carried out in relation to the "diazo reaction" of the urine of typhoid patients, it was found desirable to submit the urine to Kutscher's process for the isolation of bases. The process, as is well known, consists in fractional precipitation by means of silver, first in acid and subsequently in alkaline solution. Treatment of the urine by this means resulted in the isolation of an unexpectedly large amount of methylguanidine. Thus, while previous workers, Kutscher and Lohmann [1906], Engeland [1908] and Achelis [1906], had obtained varying amounts of methylguanidine which corresponded to an average yield of 0·01 g. of base per litre, the average amount obtained in the experiments under consideration was approximately 0·06 g. per litre, and in one case 0·17 g. per litre was isolated.

It was at first thought that this result was of some pathological significance, but in order to confirm the earlier results recorded in the literature, a quantity of normal urine was submitted to a process of fractionation similar in all respects to that carried out upon the typhoid urine. The result was somewhat unexpected; an amount of methylguanidine being obtained approximately corresponding to 0·07 g. per litre.

From this result it appeared either that the amount of methylguanidine present in normal urine had been underestimated, owing to incomplete isolation, or that under the conditions of fractionation as carried out in the above experiments methylguanidine had been produced from some other substance present in the urine.

It is at once evident that the last hypothesis is by no means improbable. Methylguanidine, as is well known, is readily obtainable from creatinine (or creatine) by oxidation, either by boiling with mercuric oxide in acid solution, as first shown by Dessaignes [1854], or according to Neubauer [1861] by permanganate. It seemed therefore by no means unlikely that in the presence of silver oxide produced in the fractionation, a certain amount of the creatinine of the urine would be oxidised in a similar manner giving rise to methylguanidine. The larger yield of the latter base obtained in the present instance would readily be explained by the fact that in order to avoid precipitation of creatinine as far as possible, a large excess of baryta solution was employed and the precipitated solution allowed to stand for some considerable time in accordance with the process employed by Engeland [1908]. Experiments were therefore carried out in which pure creatinine was submitted to the conditions obtaining in the process of fractionation. To the solution of pure creatinine, silver nitrate was added until such an excess was present that on addition of baryta an immediate brown precipitate (which rapidly became black) was produced. Warm saturated baryta solution was then added in excess. After suitable treatment methylguanidine was readily isolated as a crystalline picrate. Further, it was shown that, according to the conditions under which the fractionation was carried out, larger or smaller amounts of methylguanidine were produced. Thus, working as rapidly as possible at a low temperature, only a very small amount of methylguanidine was obtained, while if the solutions used were warm, larger quantities were produced. By heating creatinine with silver nitrate and baryta in excess on the water bath for an hour or so, a nearly quantitative yield of methylguanidine can be obtained, this method indeed being perhaps the most suitable for the preparation of the base in quantity.

In view of the foregoing results, it would appear that the statements found in the literature with regard to the occurrence of methylguanidine in urine, muscle, etc. must be looked upon with some distrust, at any rate in those cases where the base has been isolated by the Kutscher process.

According to Kutscher [1905] and Gulewitsch [1906] commercial meat extracts contain large amounts of methylguanidine (0·38 % of the nitrate according to Gulewitsch), but since the base was isolated as described above, and warm baryta solution was employed, the figures obtained were at least considerably too high. Krimberg [1906] again obtained methylguanidine from fresh horse meat by the silver fractionation, whereas Brieger [1886] using another method was only able to obtain it from horse meat after putrefaction. Smorodinzew [1913] obtained 0·083 % from fresh horse meat and also [1912] found the base in liver. It is to be noticed however that by a preliminary fractionation of the phosphotungstic precipitate from extract of horse meat he obtained two fractions, one of which contained creatine, the other being creatine-free. From the former fraction only could methylguanidine be obtained.

The amounts which have been isolated from normal urine are in all cases small. It is perhaps significant that Achelis [1906] failed to isolate any methylguanidine from normal urine until the fractionation had been repeated once or twice (*i.e.* only after somewhat prolonged treatment with silver and baryta). On the other hand, Kutscher and Lohmann [1906] obtained small amounts by another process of fractionation. The statement by Koch [1912] that a large amount of methylguanidine (1·9 g. of the gold salt per litre) occurs in the urine of parathyroidectomised dogs appears to be beyond criticism since the method of isolation employed did not involve the use of silver and baryta.

Heyde [1911] has also stated that the urine of animals killed by anaphylactic shock contains a large amount of methylguanidine, but no details of the method employed for its isolation are given.

EXPERIMENTAL.

Fractionation of Urine. (A.)

To 1420 cc. of urine, which in this case was that of a typhoid patient, were added 380 cc. of a 20 % aqueous solution of normal lead acetate. The precipitate was filtered off and the filtrate concentrated to about 100 cc. The concentrated solution was neutralised and then completely precipitated by alternate addition of a hot saturated aqueous solution of mercuric chloride and a saturated solution of sodium acetate until a portion of the filtrate no longer gave more than an opalescence on further addition of either reagent. The precipitate was filtered off, suspended in dilute hydrochloric acid solution and digested on the water bath until the reaction remained permanently acid to Congo red. The insoluble portion was removed by filtration and discarded. The acid filtrate was decomposed by sulphuretted hydrogen, the mercuric sulphide filtered off, the filtrate freed from sulphuretted hydrogen by a current of air, neutralised with caustic soda and evaporated to dryness *in vacuo*. The residue was completely extracted with hot methyl alcohol, the solvent distilled off under reduced pressure and the residue taken up in a little water. The aqueous solution was made just acid to Congo red with dilute nitric acid and a 20 % aqueous solution of silver nitrate added until precipitation was complete. This precipitate (containing the purine bases) was filtered off and set aside.

To the filtrate (the volume of which was 150 cc.) was then added silver nitrate until the solution gave an immediate brown precipitate with baryta solution. Warm saturated baryta solution was then added in excess and some additional solid baryta also stirred into the solution, in order to ensure the decomposition of the creatinine silver compound. After 24 hours the precipitate was filtered off, re-dissolved by addition of dilute nitric acid, filtered from insoluble material, and again precipitated with excess of baryta solution. After standing, the precipitate was separated by centrifuging, washed by suspending in water and again centrifuging and finally dissolved in dilute nitric acid and filtered. The clear acid filtrate was then treated with dilute aqueous ammonia until a little of the filtered solution gave no precipitate with ammoniacal silver nitrate. The yellowish white precipitate was filtered off and worked up in the usual way.

The filtrate was completely precipitated by addition of saturated baryta solution. The precipitate was filtered off, washed well with water, suspended in water and decomposed by sulphuretted hydrogen. The filtrate was evaporated to small bulk and on treatment with hot saturated picric acid solution gave a crystalline picrate which on recrystallisation yielded 1·05 g. of pure methylguanidine picrate, melting at 201–202°. The identity of the base was established by direct comparison of the picrate with methylguanidine picrate obtained from another source and by direct conversion into the crystalline nitrate.

In another experiment in which 7·7 litres of typhoid urine were fractionated under similar conditions except that the treatment with silver and baryta was less prolonged, a final yield of 2·05 g. of methylguanidine picrate was obtained.

Fractionation of urine. (B.)

4,600 cc. of normal urine were fractionated exactly as described above. The yield of pure methylguanidine picrate was in this case 1·3 g.

Conversion of creatinine into methylguanidine.

1·8 g. of creatinine obtained by decomposition of the creatinine zinc salt ($Kr_2SO_4ZnSO_48H_2O$) [Folin and Blanck, 1910] was dissolved in water and the solid made acid to Congo red with dilute nitric acid. A 20 % aqueous solution of silver nitrate was then added until a brown precipitate was immediately obtained on treating a drop of the solution with baryta. Warm saturated baryta solution was then added in excess and the mixture allowed to stand over night. The precipitate was then filtered off, washed, redissolved in dilute nitric acid and the process repeated. The precipitate so obtained was dissolved in dilute nitric acid and fractionated. Dilute aqueous ammonia was added until no precipitate was obtained on addition of ammoniacal silver nitrate to the filtered solution. The precipitate so formed was filtered off and the filtrate precipitated by addition of bartya solution in excess. The precipitate was suspended in water decomposed by sulphuretted hydrogen, freed from traces of barium and concentrated. On addition of hot saturated picric acid to the concentrated solution, 0·6 g. of a crystalline picrate melting at 201–202° was obtained which was identified as methylguanidine picrate.

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IX. THE STABILITY OF PANCREATIC LIPASE.

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(*Received February 3rd, 1916.*)

For some years glycerol extracts of pancreas have been extensively used in this laboratory by Rosenheim, Shaw-Mackenzie and others. The lipase contained in the extracts has been the centre of interest, but the presence of other enzymes and the bearing this may have on the behaviour of lipase have not been overlooked. The most important of these enzymes, in this respect, is trypsin, which has often been shown to be present in these glycerol extracts.

Conflicting statements with regard to the action of trypsin on lipase appear in the literature. Experiments with pancreatic juice carried out by Terroine [1910] and by Mellanby and Woolley [1914] show that lipase rapidly disappears in presence of trypsin. Mellanby and Woolley state further that glycerol extracts of pancreas contain trypsinogen and enterokinase, and that activation is inhibited by the glycerol; but that dilution removes this inhibitory influence and trypsin is formed which destroys the lipase present in the extract. Shaw-Mackenzie [1915], on the other hand, claims that trypsin is present in the concentrated extract, and Tsuji [1915] has shown that dilution not only does not abolish, but may even increase, the lipoclastic activity.

These contradictory statements leave us in a state of uncertainty as to the stability of the lipase in glycerol extracts of pancreas. All the extracts used in this laboratory have been strongly lipoclastic and those tested have been found to be proteoclastic. An extract has been employed for class work for the last two years and has suffered no deterioration in its fat-splitting power during that time. Dr Shaw-Mackenzie has kindly supplied me with notes showing that some of the extracts prepared by Rosenheim and himself were active more than two years after they were made. As several of these extracts have been kept, I have taken the opportunity to investigate further the stability of the lipase.

EXPERIMENTAL.

Preparation of the extracts.

All the extracts were prepared in the same way. Fresh pig's pancreas was freed from fat, minced and weighed. It was then allowed to stand for 24 hours with glycerol, 2 cc. of glycerol being used for 1 g. of pancreas. The extract was strained off through two layers of muslin, and kept in a stoppered bottle. Just before use one part of this concentrated extract was diluted with two parts of water.

Estimation of lipoclastic activity.

The method employed was that used by Rosenheim and Shaw-Mackenzie [1910]. 5 cc. of an olive oil emulsion were incubated with 3 cc. of the dilute extract at 37° for 18–24 hours and the fatty acids estimated by titration with N/10 NaOH, alcohol being added to facilitate titration and phenolphthalein being the indicator. The estimations were done in duplicate and the means of the results were corrected for the original acidity of the mixture of emulsion and extract. The details of the method have recently been very fully described by Umeda [1915]. I find, with him, that the quality of the oil is of importance. The length of time during which the mixture of extract and emulsion is shaken has also to be considered. Bradley [1909] has investigated the effect of shaking emulsions, and his results show that, if an emulsion be shaken too long, lipoclasis is lessened. He suggests in explanation that aëration destroys the enzyme or makes it difficult for the enzyme to reach and act upon the oil. While this may be the explanation in many cases, some of my experiments show that, in shaking the mixture of extract and emulsion, a difference in time of only ten seconds causes the oil to separate out, thus producing a condition which should retard lipoclasis. In the following experiment it will be seen that shaking for ten seconds gave a much better result than shaking for twenty seconds.

Exp. 1. Flasks containing 3 cc. of dilute extract and 5 cc. of oil emulsion were shaken for 5, 10, and 20 seconds, respectively, and put into the incubator at 37° for 22 hours. The mixture, before incubation, required 2 cc. of

N/10 NaOH to neutralise it. This number was subtracted from those obtained after incubation, and the corrected numbers are given.

Time of shaking, secs.	N/10 NaOH cc.
5	32·8
10	35·2
20	20·4

The oil had separated out in the last flask only. With an oil resistant to lipase this difference is not appreciable as the numbers obtained are all small. This may be due to the fact, pointed out by Umeda [1915], that the oil contains an impurity which resists the action of lipase even when the oil is finely emulsified. Such oils, too, often form thin emulsions, and when mixed with the extract they separate out in the incubator, or, more rarely, on being shaken for a short time. The inappreciable differences produced by shaking, and the low numbers obtained with these oils, are evident on comparing the following with the preceding experiment, the extract used being the same in both cases.

Exp. 2. Pairs of flasks containing 3 cc. of dilute extract and 5 cc. of oil emulsion were shaken for 5, 10, and 20 seconds, respectively, and put into the incubator at 37° for 18 hours. The mixture, before incubation, required 1·1 cc. N/10 NaOH to neutralise it. The mean of the titration numbers for each pair, after incubation, was taken, and these means were corrected for the original acidity. The final numbers are given.

Time of shaking, secs.	N/10 NaOH cc.
5	3·3
10	3·4
20	3·1

In order that differences due to shaking might not vitiate the experiments, the mixture of extract and emulsion was shaken slowly ten times in all the subsequent work.

Estimation of tryptic activity.

The tryptic activity was measured by Sörensen's [1908] formaldehyde method, decinormal sodium hydrate being used for titration.

The following was the usual procedure:

3 cc. of dilute pancreatic extract and 10 cc. of a 2 % solution of caseinogen in 0·4 % sodium carbonate were put into each of three flasks. The amino-acids were at once estimated in one of these by the addition of 10 cc. of neutral formalin and subsequent titration with N/10 NaOH. The other two

flasks, after the addition of 0·5 cc. of toluene to each, were put into the incubator at 37° for 18–24 hours. The amino-acids were then estimated in the same way. The mean of the numbers obtained for these two was taken, and from it was subtracted the number obtained for the control flask. The result was a measure of the proteoclastic activity.

The activity of old extracts.

Ten extracts which had been prepared at periods varying from nearly six to three years ago were examined, and their lipoclastic and proteoclastic powers compared with those of an extract three months old (No. 11 in the series) and known to be active. The extracts were diluted before use for the lipoclastic but not for the tryptic determinations, and the experiments were carried out in the manner already described, lipoclasis going on at 37° for 18 hours, and proteoclasis for 24 hours. Each experiment was performed in duplicate and the mean of the results corrected for the original acidity of the mixture of extract and substrate. For the numbers illustrating the lipoclastic activity (after 18 hours at 37°) of the extracts soon after preparation I am indebted to Dr Shaw-Mackenzie. The proteoclastic activity at that time was not recorded. The numbers are collected together in the following table.

	Lipoclastic activity in cc. N/10 NaOH		Time interval between (I) and (II)		Tryptic activity in cc. N/10 NaOH
No.	(I)	(II)	yrs.	mths.	
1	14·8	31·2	5	8	6·3
2	23·6	26·6	5	7	4·9
3	28·9	20·7	5	4	3·9
4	16·8	15·9	5	4	3·9
5	12·6	5·2	5	4	2·6
6	14·1	24·1	5	3	3·7
7	28·1	26·5	5	2	5·4
8	13·3	26·6	4	10	4·3
9	39·2	15·5	3	11	3·8
10	25·8	27·6	3	4	5·4
11	—	30·0	—	—	7·4

(I) shows the original and (II) the present lipoclastic activity. The numbers in (I) are not strictly comparable with each other as the estimations were not all made with the same oil; those in (II) are comparable with each other, having all been obtained under the same conditions, but are not strictly comparable with those in (I). It is evident, however, that all the extracts are still lipoclastic and, except No. 5, compare favourably with the recent preparation No. 11. No. 5 is a thin extract and I suspected that it had been diluted, but Dr Shaw-Mackenzie's notes showed that it was made in the usual way, and there is no reason to believe that it has since been tampered with. It is evident, too, from the last column, that all these extracts are tryptic. Mellanby and Woolley [1914] state that glycerol extracts of pancreas are tryptic only on dilution. Shaw-Mackenzie [1915] has shown, by the serum plate method, that the *concentrated* extract is tryptic. I am able to confirm this, for, with the serum plate method, all our concentrated extracts are tryptic. Concentrated glycerol extracts of pancreas, therefore, can be kept for a considerable time and still retain lipoclastic and proteoclastic activity, the glycerol apparently inhibiting the destructive action of trypsin on lipase.

The stability of dilute extracts.

For most of our work the concentrated extract was diluted with twice its volume of water shortly before use. To what extent this dilute extract retains lipoclastic and proteoclastic activity after it has been kept for some time, has been the subject of further investigation.

The following preparations were made:

A.	Concentrated glycerol extract ...	67 cc.
	Distilled water	133 „
B.	Concentrated glycerol extract ...	67 „
	1 % solution of formaldehyde ...	10 „
	Distilled water	123 „

(= 1 in 2000 formaldehyde)

The lipoclastic and proteoclastic activities of these preparations were at once examined in the usual way. The flasks containing them were corked and left at room temperature for 5 days when a second examination was made. To A, from which 18 cc. in all had been removed, 2 cc. of toluene were added. Both flasks were corked and left at room temperature. Forty-six days after the date of preparation they were again examined. In all the examinations lipoclasis and proteoclasis went on for 24 hours in the incubator at 37°. The results are here given.

		A	B
Lipoclastic activity in cc. N/10 NaOH	at once	30·9	29·8
	after 5 days	30·1	29·1
	after 46 days	29·3	25·1
Tryptic activity in cc. N/10 NaOH	at once	5·1	4·8
	after 5 days	4·2	4·2
	after 46 days	3·2	3·3

The tryptic power of both had diminished to some extent, but in spite of the presence of trypsin both preparations were still strongly lipoclastic, the activity of the toluene preparation being practically unimpaired while that of the formaldehyde preparation was slightly diminished. The preparations gave no odour of putrefaction.

Even with greater strengths of formaldehyde the extracts retain both lipoclastic and proteoclastic activity.

The following preparations were made:

No.	Concentrated extract cc.	Distilled water cc.	1 % formaldehyde solution cc.	Toluene cc.
I	100	200	—	—
II	100	200	—	4
III	100	170	30	—
IV	100	140	60	—

The strength of formaldehyde in III was 1 in 1000 and in IV 1 in 500.

The lipoclastic and proteoclastic activities were examined in the usual way, incubation going on for 22·5 hours at 37°. The flasks containing the preparations were corked and left at room temperature. After 42 days No. I contained a mould (*Penicillium glaucum*) but there was no odour of any kind, No. II gave the odour of toluene, and Nos. III and IV had an oily but not putrid odour.

In every case, as also in the last experiment, a precipitate had separated out and risen to the top. The mixture was therefore always shaken before samples were removed for examination.

The activities just after preparation and 42 days later are given.

		I	II	III	IV
Lipoclastic activity in cc. N/10 NaOH	at once	23·4	23·4	22·7	23·7
	after 42 days	23·4	28·9	16·4	13·1
Tryptic activity in cc. N/10 NaOH	at once	5·5	5·5	5·3	4·9
	after 42 days	5·1	5·3	5·0	4·6

All were still lipoclastic and proteoclastic. The proteoclastic activity showed no appreciable change. The lipoclastic activity was diminished in the extracts containing formaldehyde but gave a higher number in that containing toluene. The activity of the extract without antiseptic was in no way impaired. It is interesting to note that formaldehyde, in the strengths used, did not destroy the lipase.

Thus it appears that in these dilute extracts trypsin and lipase can exist together without destruction of the latter, the extracts containing enough glycerol to protect the lipase.

CONCLUSIONS.

1. Glycerol extracts of pancreas retain their lipoclastic and proteoclastic activity even after more than five years.

2. Dilute extracts (1 in 3), kept with or without antiseptics for six weeks, show both lipoclastic and proteoclastic activity.

3. Trypsin and lipase are present together in concentrated and dilute glycerol extracts, the glycerol apparently inhibiting the destructive action of trypsin on lipase.

I wish to thank Dr Rosenheim for suggesting to me the subject of this research and for much kind assistance.

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X. THE ANALYSIS OF PROTEINS. I. THE ESTIMATION OF ARGININE BY DECOMPOSITION WITH ALKALI.

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In the procedure suggested by van Slyke [1911] for the analysis of proteins, the arginine, which is precipitated together with the histidine, lysine and cystine by phosphotungstic acid, is estimated by boiling the solution containing these four compounds with 50 % caustic potash for six hours. Under these conditions the guanidine grouping of the arginine is decomposed with the formation of ammonia, which is collected in excess of standard acid and determined by titration. Twice the amount of the nitrogen as ammonia corresponds to the amount of arginine according to the equation:

$$\begin{aligned}&H_2N\,(NH)\,C\cdot NH\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH\,(NH_2)\cdot COOH + 2H_2O\\&\quad = 2NH_3 + CO_2 + H_2N\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH\,(NH_2)\cdot COOH.\end{aligned}$$

In carrying out analyses of proteins by this method difficulty has been experienced in obtaining data for the amount of arginine. Numerous determinations have been lost through the action of the strong caustic alkali upon the glass vessels and duplicate analyses have not agreed with one another. Further, results obtained by the substitution of a copper vessel in which the alkaline decomposition was carried out were generally higher than those obtained with glass vessels. It became necessary therefore to enquire more closely into the decomposition of arginine and the other compounds by caustic alkali.

Previous workers have not described any detailed experiments upon the action of alkali upon arginine. Schulze and Likiernik [1891] described the formation of urea by boiling arginine with saturated baryta water and of ammonia and carbon dioxide by heating arginine under pressure with baryta water. Osborne, Leavenworth and Brautlecht [1908] found that arginine was slowly decomposed by distillation with 0·1 N sodium hydroxide, and van Slyke [1911] makes no other statement than that arginine is completely decomposed by boiling with 50 % caustic potash (12·5 g. KOH to 25 cc. solution) for six hours.

Cystine, as is well known and as was pointed out by van Slyke, is decomposed by boiling with alkali, but its amount in proteins other than the keratins is usually so small that the amount of ammonia evolved from it is negligible: in the case of the keratins van Slyke gives a correction.

Lysine is a diamino acid and is stable to alkali.

Histidine contains a glyoxaline ring in its molecule and it is possible that this ring is broken down with liberation of ammonia by the drastic treatment with caustic alkali.

(1) *Action of Caustic Alkali upon Arginine.*

The arginine used in these experiments was prepared by the usual method from gelatin and isolated as carbonate. This salt, instead of the nitrate, was chosen so as to avoid any possibility of the action of nitrates upon the decomposition, but it is more difficult to obtain in a pure state. The recrystallised preparation consisted partly of carbonate and partly of free base as shown by its nitrogen content of 26·2 %; the free base contains 32·0 % and the carbonate 23·7 %.

Solutions of this preparation of about 0·1 % concentration were gently boiled with caustic soda of various strengths for periods of 6 to 11 hours either in a glass flask of about 200 cc. capacity or in a copper flask of about 400 cc. capacity; to the flask was attached a condenser and to the condenser a U-tube of the pattern devised by Folin. The experiments were carried out exactly as described by van Slyke.

The results were as follows:

(*a*) *With the copper flask.*

Concentration of NaOH %	Time of boiling hours	NH_3 evolved in cc. 0·1 N	One-half total nitrogen in solution in cc. 0·1 N
10	6	9·2	11·4
50	6	27·4	28·5
20	6	10·7	11·4
20	8	8·3	8·7
20	8	7·6	8·0

(*b*) *With a glass flask.*

	Concentration of NaOH %	Time of boiling hours	NH_3 evolved in cc. 0·1 N	One-half total nitrogen in solution in cc. 0·1 N
I	4	6	17·2	28·5
	10	6	8·8	11·4
	20	6	10·6	11·4
II	20	8	7·8	8·0
	50	6	7·3	8·0
	20	6	7·3	8·0
	20	6	7·3	8·0
	20	6	7·3	8·0
III	20	7	3·2	2·8
	20	7	2·5	2·8
	20	6·5	3·2	2·8
	20	6·5	2·7	2·8

The experiments were performed in three groups with different quantities of total nitrogen in solution. In the first group it was found at once that 4 and 10 % caustic soda did not give an amount of ammonia corresponding to half the amount of arginine nitrogen in solution, and that 20 % gave nearly the correct amount. In the second group confirmation of the previous result was obtained; the same result was given when 50 % caustic soda was used. Further confirmation is given by the experiments in the third group. In all experiments an amount of ammonia corresponding to half the total was never obtained. This was probably due to impurity in the preparation of arginine, which was not purified by conversion into the nitrate or the copper nitrate double salt. The experiments show that the estimation of arginine can be effected by boiling with 20 % caustic soda in place of 50 %.

In practice it is more convenient to add an equal volume of 40 % caustic soda to the solution than to weigh out the correct quantity of soda; in this way also a larger volume of liquid is contained in the flask. After boiling for six hours it is necessary according to van Slyke's directions to disconnect the parts of the apparatus and distil off the remaining ammonia which does not diffuse into the U-tube. Loss of ammonia may thus occur; it is sometimes noticeable by its smell. By using an equal volume of 40 % sodium hydroxide and consequently more liquid in the flask, it is not necessary to disconnect the parts of the apparatus; the ammonia remaining in the flask and condenser can be distilled directly into the U-tube if the water be run out of the condenser and the boiling continued for another 15 to 20 minutes.

The same results are obtained when the decomposition is carried out in a copper flask, but as will be seen from the data given by histidine under the same conditions it is not possible to use a copper flask.

(2) *Estimation of the Total Nitrogen of the Bases in the Solution.*

In van Slyke's procedure the total nitrogen of the arginine and of the other compounds in the solution is determined in the residue remaining after the arginine estimation. Owing to the presence of silica in the solution, dissolved by the alkali from the glass, bumping occurs and nitrogen is lost. This is lessened by using 20 % sodium hydroxide in the arginine estimation but is not eliminated; it is avoided by using a copper flask, but as previously stated a copper flask cannot be used.

It is advisable to estimate the total nitrogen in a separate portion (5 cc.) of the solution of the bases rather than in the residue.

(3) *Action of Caustic Alkali upon Histidine.*

The histidine used in these experiments was prepared by the usual method from defibrinated blood and isolated as hydrochloride. The preparation gave the following figures on analysis:

$$H_2O = 8{\cdot}55;\ N = 19{\cdot}8;\ Cl = 16{\cdot}7\ \%.$$

Calculated for

$$C_6H_9O_2N_3HCl\,.\,H_2O;\ H_2O = 8{\cdot}59;\ N = 20{\cdot}05;\ Cl = 16{\cdot}94\ \%.$$

20 or 25 cc. of approximately 0·5 % histidine hydrochloride solution were boiled with caustic soda of varying concentration in a glass or copper flask in the same way as described above in the case of arginine. The results were as follows:

(*a*) *With the copper flask.*

Concentration of NaOH %	Time of boiling hours	NH_3 evolved in cc. 0·1 N	Total nitrogen of histidine solution in cc. 0·1 N
50	6	3·3	14
50	6	4·6	23
25	6	1·9	13
25	11	2·1	13
20	8	1·9	13

(*b*) *With a glass flask.*

Concentration of NaOH %	Time of boiling hours	NH_3 evolved in cc. 0·1 N	Total nitrogen of histidine solution in cc. 0·1 N
50	2*	0·3	14·0
50	6*	0·3	14·0
50	6	0·4	23·0
20	6	0·5	13·0

* In these experiments the glass flask was destroyed by the alkali and the ammonia given in the table had diffused into the standard acid.

Histidine is thus decomposed by boiling with caustic alkali. The amount of decomposition in the cases in which a copper flask was used corresponds to about one-fourth of the total nitrogen of the histidine but the figures are not exact multiples and vary considerably. The amount of decomposition in the experiments in which a glass flask was used is very much less and corresponds to from 1·5 to 3 % of the total nitrogen in the solution.

This slight decomposition of histidine may however be neglected in the estimation of arginine; to some extent it makes up for the small loss which occurs in the determination of the arginine.

SUMMARY.

The estimation of arginine can be effected by boiling with 20 % sodium hydroxide, instead of 50 %, for six hours. The small loss of ammonia which occurs is counterbalanced by the slight decomposition of histidine which takes place when a solution of bases as obtained by van Slyke's method is being analysed.

The estimation cannot be effected in a copper flask if histidine is present, as under these conditions histidine undergoes considerable decomposition. It is advisable, on account of bumping with consequent loss of nitrogen, to estimate the total nitrogen of the arginine and other bases in a fresh portion of the solution rather than in the residue from the arginine estimation.

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XI. ON THE INOSITOL OF BRAIN AND ITS PREPARATION.

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(Received January 19th, 1916.)

Shortly after Scherer's [1850] discovery of inositol in muscle, von Bibra [1854] carried out a careful but unsuccessful search for this substance in brain. The presence of inositol in brain was first demonstrated by W. Müller [1857], and L. W. Thudichum [1884] is the only author who has since given attention to its occurrence.

The preparation of inositol from brain (and other organs) presents certain difficulties owing partly to the small amounts present and partly to the lack of trustworthy methods. The usual procedure, which has not varied much since Scherer [1850] first used it in the case of muscle, consists in extracting the organs with water and in precipitating the inositol from the suitably purified extract as an insoluble metallic compound by means of either basic lead acetate, copper acetate or baryta. From its combination with lead, copper or barium the inositol is set free by means of sulphuretted hydrogen or by sulphuric acid and finally crystallises out from the concentrated solution on the addition of alcohol.

W. Müller [1857] ground the brain with neutral lead acetate and water, allowed the mixture to stand and thus obtained a blood-red extract, which could be easily filtered and from which he was able to isolate inositol by means of basic lead acetate. The objection to this method is that it offers no guarantee of the complete extraction of the inositol, which moreover is usually obtained in a very impure condition in the first instance.

The difficulty encountered by von Bibra [1854] of filtering a watery extract of brain is easily overcome by gradually introducing the finely minced brain into boiling water and by acidifying with acetic acid before filtration. It was to be expected that by a repeated extraction under these conditions practically the whole of the inositol would be removed. To my surprise however I found that when working up 1 kilo. of fresh ox-brain in this way, I was unable to obtain even a trace of inositol.

This result seems at first sight to be in favour of the view of F. Rosenberger [1908], according to which inositol does not occur as such in fresh organs, but is formed post-mortem by an autolytic or enzymic process from a precursor called "inositogen." This hypothetical substance was, however, not isolated as such and its existence was only inferred from the failure to isolate inositol itself. Rosenberger elaborated a method for the isolation of inositol from organs which is open to serious objections. He made use of the resistance of inositol to strong alkali in order to set it free from the tissues in a way similar to that used for the estimation of glycogen. The tissues are boiled with potash until completely dissolved and the solution is concentrated to one-third of its original volume after the addition of an excess of strong nitric acid. Hot baryta is next added and the heating continued for fifteen minutes, after which time the solution is again acidified and concentrated to a syrup. This is heated with concentrated nitric acid until the violent reaction is over, and again neutralised with baryta. If necessary the treatment with nitric acid and baryta is repeated several times. The final solution is cleared by neutral lead acetate and from the filtrate any inositol, which has survived this prolonged treatment, is precipitated by basic lead acetate in the usual way.

The negative results so often yielded by this method may well be due to the destruction and oxidation of inositol by the repeated treatment with strong nitric acid, as has been pointed out by E. Starkenstein [1908]. Moreover, the presence of the large quantities of salts introduced during the process may easily lead to the loss in the first lead precipitate of the small quantities of inositol normally present. It had already been noted by Thudichum [1884] that under certain conditions neutral lead acetate may carry down inositol and Meillère [1906] has traced this to the presence of salts.

It is not unlikely that von Bibra's and my own failure to isolate inositol from a brain extract made with boiling water is due to the same reason. I have not examined this subject any further, but as I was able to isolate inositol, by a method to be described, from the same lot of brain which failed to yield a trace by the usual extraction method, I incline to the view that as far as the brain is concerned the existence of "inositogen" is very unlikely.

I finally succeeded in finding a method which enables the preparation of inositol from brain to be made with certainty by making use of an observation kindly communicated to me by Dr O. Rosenheim. In the course of the method introduced by him [1906, 1914] for the preparation of lipoids from brain, acetone was used in the first instance for the dehydration of brain and the preparation of cholesterol. Rosenheim observed that the first watery acetone extract prepared at room temperature was free from cholesterol and that from it inositol could be easily prepared.

I was able fully to confirm this observation and found that this method of extraction of inositol leads more quickly to a pure product than W. Müller's original method and that the yield also is greater. I have not yet applied the method to any other animal organs or plants, but it is to be expected that it might be found useful in this case also, especially as the amount of inorganic salts, which interfere with the preparation of inositol, is considerably decreased when acetone is used as a solvent.

In his work on inositol, Thudichum [1884] suggests that the inositol from human brain "is either altogether different from that contained in the brain of the ox, or is accompanied by another similar carbohydrate of less stable quality." This conclusion was arrived at by the analysis of the copper compounds of a well-crystallised and apparently perfectly pure specimen of inositol from human brain, which contained up to 6 % more copper than the corresponding compounds prepared from bovine inositol.

This statement of Thudichum's, so many of whose originally discredited observations have since been confirmed, warranted further investigation. It is now known that inositol may exist in several optically isomeric modifications, although its accepted formula does not contain an asymmetric carbon atom. It was pointed out by O. Aschan [1902] that van't Hoff and Le Bel's theory allows the existence of seven inactive forms besides the two active ones. Of these *d*- and *l*-inositol are known to occur as methyl esters in plants, whilst the ordinary inositol of animals and plants represents one of the seven inactive forms. It has, however, recently been shown by Johannes Müller [1907] that a second inactive modification of inositol is represented by scyllitol, a substance discovered by Staedeler and Frerichs [1858] in the kidneys, liver and other organs of certain plagiostomous fishes (i.e. elasmobranchs such as the shark, dogfish, etc.). The same substance occurs also in acorns, originally called "quercine" by Vincent and Delachanal [1887] and in the leaves of coconut palms (*Cocos nucifera* and *Cocos plumosa*) from which it was isolated as "cocositol" by Hugo Müller [1907, 1912]. The two inactive inositols resemble each other in their solubilities and chemical properties, but possess melting-points differing by as much as 125°.

I prepared inositol from brain by the method described above and from a comparison of their physical constants I came to the conclusion that the inositol occurring in human brain is identical with that of ox brain and the ordinary *i*-inositol. Thudichum's analytical figures therefore only prove that the copper compounds of inositol are of varying composition and not suitable for the identification of inositol.

EXPERIMENTAL.

(1) *The preparation of inositol from brain.*

Several attempts to isolate inositol by the usual methods from a brain extract made with boiling water were unsuccessful, not even enough material for a colour reaction being obtainable. Through Dr de Souza's kindness an acetone extract of 10 kilo. of ox-brain, which he did not require for the purpose of his work, was put at my disposal. This was freed from acetone by distillation and the remaining watery extract filtered. To the filtrate neutral lead acetate was added in excess. The resulting precipitate was filtered and washed. To the filtrate basic lead acetate and ammonia were added, the precipitate again filtered, suspended in hot water and decomposed with sulphuretted hydrogen. The lead-free filtrate was concentrated to a small bulk and alcohol added to the hot solution until crystallisation of inositol began. The crude inositol, which weighed about 4 g., was recrystallised from water and a well-crystallised product, showing a melting-point of 225°, was easily obtained. Having made myself familiar with the properties of inositol, I proceeded to compare the new acetone method with that of W. Müller's [1857] by which he originally obtained inositol from brain and with the method of Scherer [1850] which is successful in the case of muscle.

For this purpose a large quantity of fresh ox-brain was passed through a mincing machine. Three portions of 1 kilo. each were weighed out and worked up by different methods.

(*a*) *Müller's method.* One kilo. of minced brain was ground with solid lead acetate and one litre of water into a thin emulsion. After allowing the mixture to stand for about 18 hours a clear blood-red solution had separated from the brain mass, and this was filtered through muslin. The solution was boiled and filtered again. Basic lead acetate and ammonia were now added, the bulky precipitate allowed to settle for some hours and filtered. According to my experience it is not advisable to wash this precipitate with distilled water, as the inositol-lead compound seems to dissociate on prolonged washing. It may, however, be washed with a solution of ammonium carbonate. The precipitate is next suspended in hot water and decomposed with sulphuretted hydrogen. The lead-free solution is concentrated to a small bulk (about 20–30 cc.) and absolute alcohol added until the crystallisation of inositol begins. After standing for 24 hours in the ice chest, the inositol is filtered and washed with alcohol and ether. The yield of crude, rather yellowish inositol was 0·22 g.

(*b*) *Scherer's method.* One kilo. of minced brain was gradually introduced into one litre of boiling water and acidified with acetic acid. After cooling a clear filtrate was easily obtained. The extraction with boiling water was repeated once more. The combined filtrates were precipitated with neutral lead acetate, the precipitate filtered and well washed. The filtrate from the neutral lead acetate was treated with basic lead acetate and ammonia as before. The final solution remained perfectly clear on the addition of absolute alcohol and I was unable to separate from it any substance giving the colour reactions typical for inositol.

(*c*) *Acetone method.* One kilo. of minced brain was well shaken up with one litre of acetone, left standing for about 18 hours and strained through several layers of muslin. The treatment with acetone was repeated, the combined extracts heated to boiling and filtered. From the clear yellowish filtrate the acetone was removed partly by distillation and finally, after the addition of water, on the water-bath. The watery solution was treated with lead acetate as before. The final lead precipitate was much less bulky than those obtained in the previous methods and the inositol, crystallising out on the addition of alcohol, was nearly white. The yield of crude inositol was 0·32 g.

(2) *The identity of the inositol of human and ox brain.*

A sample of inositol from human brain was prepared by the acetone method above described. If Thudichum's [1884] suggestion that the inositol of human brain is not identical with that of ox brain were correct, we should expect the inositol from human brain to represent either one of the optically active isomeric forms of inositol or another of the possible seven inactive forms. In both cases an examination of the behaviour towards polarised light and a determination of the melting-point would form a sure criterion. The melting-point of scyllitol, the only other known form of *i*-inositol, is 350° [H. Müller, 1912], whilst ordinary inositol melts at 225°.

I examined 10 % solutions of inositol prepared from human and ox brain in a large Lippich-Polarimeter (with three field division) and failed to find in either case any optical activity.

The melting-point of two carefully purified specimens was 225°. A mixed sample showed no alteration of melting-point. Another sample mixed with *i*-inositol prepared from seeds (phytin) fused also at 225°.

Both preparations possessed the same solubilities and crystalline form and gave Salkowski's [1910] modification of Scherer's reaction, alike qualitatively as well as quantitatively.

The inositol of human brain agrees in its behaviour towards polarised light, in its melting-point and colour reaction with the inositol of ox brain, and both are identical with the ordinary *i*-inositol of plants.

The expenses of this investigation have been defrayed out of a grant made to Dr Rosenheim from the Government Grant Committee of the Royal Society.

CONCLUSIONS.

1. A trustworthy method for the preparation of inositol from brain, based on its solubility in dilute acetone, is described.

2. The inositol of human brain is identical with that of ox brain and with the ordinary *i*-inositol of plants.

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XII. ON THE OPTIMAL REACTION FOR PEPSIN.

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That the action of most ferments depends upon the hydrogen ion concentration is now a well-known fact. Sörensen [1909] was the first to study the optimal hydrogen ion concentration for pepsin. He made acid albumin solution from white of egg and, after digestion of it with pepsin, determined the increase of soluble nitrogen left after precipitation by tin chloride or tannic acid. Thus he found that in short durations of digestion (½ or 1 hour) the optimal hydrogen ion concentration was about $[H^\cdot] = 2{\cdot}3 \times 10^{-2}$, in longer durations (12 hours or longer) nearly $6{\cdot}0 \times 10^{-2}$, gradually moving to the acid side. Michaelis and Davidsohn [1910] used caseinogen as substrate and found the optimal concentration $[H^\cdot] = 1{\cdot}5 \times 10^{-2}$, with only short duration of digestion (10–15 minutes). Michaelis and Mendelssohn [1914] found, using edestin as substrate, that the optimal point of this ferment is, on the average, $4{\cdot}0 \times 10^{-2}$; concentrations between $2{\cdot}0 \times 10^{-2}$ and $8{\cdot}0 \times 10^{-2}$ being usually nearly the same in effect. They found further that the optimal hydrogen ion concentration does not depend upon the kind of acid nor on the presence of salts; in the latter case, it moves slightly to the less acid side.

Sörensen made all his experiments of the same chlorine concentration. In my experiments, the NaCl concentration was kept constant. The details are as follows:

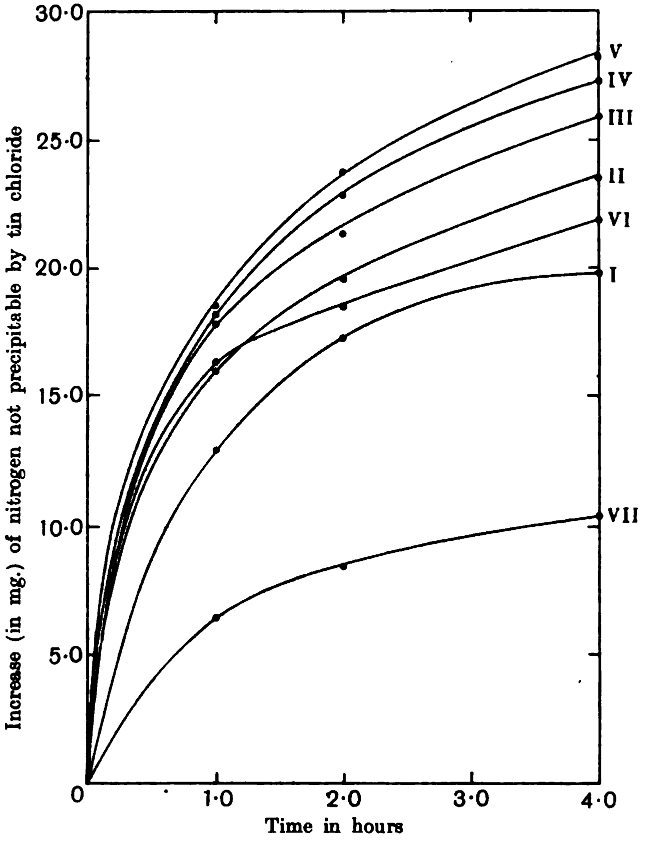
(1) 1 % *acid albumin solution as substrate.* About 3·3 % neutral protein solution was first made. This was obtained by mixing 600 cc. white of egg, 1146 cc. water and 54 cc. N/10 HCl. After shaking for two hours, this was put in the cold store until next day, when it was filtered. Then 1600 cc.

360 *cc. of approx.* 1 % *acid albumin solution* + 40 *cc. sodium hydrate solution containing sodium chloride* + 100 *cc. pepsin solution.* 30 *cc. of this mixture contained* 36·57 *mg. nitrogen, of which* 1·68 *mg. nitrogen was not precipitable by tin chloride,* 0·98 *mg. nitrogen not precipitable by tannic acid.*

Exp.	Composition of sodium hydrate + sodium chloride solution	Hydrogen ion concentration electrometrically measured		Increase (in mg.) of the amount of nitrogen which is not precipitable by tin chloride after *t* hours' digestion							Increase (in mg.) of the amount of nitrogen which is not precipitable by tannic acid after *t* hours' digestion						
		P_H	[H·]	$t=\frac{1}{2}$	1	2	4	8	24	48	$t=\frac{1}{2}$	1	2	4	8	24	48
1	40 cc. 2N NaCl	0·53 (18° C.)	$3·0 \times 10^{-1}$	8·69	12·70	17·09	19·61	24·10	29·42	30·82	1·54	2·38	4·62	5·74	9·95	—	—
2	27 cc. 2N NaCl 13 cc. 2N NaOH	0·74 (21° C.)	$1·8 \times 10^{-1}$	13·12	15·97	19·51	23·54	27·46	30·82	33·34	2·34	4·06	5·18	7·43	10·51	12·75	14·15
3	16 cc. 2N NaCl 24 cc. 2N NaOH	1·24 (22° C.)	$5·8 \times 10^{-2}$	14·15	17·93	21·30	25·78	30·54	32·22	34·18	3·01	4·34	6·02	8·40	—	14·71	15·83
4	11 cc. 2N NaCl 29 cc. 2N NaOH	1·37 (20° C.)	$4·3 \times 10^{-2}$	14·75	18·18	22·83	27·29	30·94	33·58	34·65	3·22	4·62	6·86	8·55	11·35	14·99	16·11
5	7 cc. 2N NaCl 33 cc. 2N NaOH	1·76 (19·5° C.)	$1·7 \times 10^{-2}$	15·03	18·49	23·54	28·02	31·60	34·18	34·74	2·94	4·21	5·74	8·27	10·79	14·15	15·55
6	3 cc. 2N NaCl 37 cc. 2N NaOH	2·22 (22° C.)	$6·0 \times 10^{-3}$	13·80	16·25	17·93	21·90	25·22	29·14	31·38	21·0	4·06	4·34	6·59	8·83	11·63	14·43
7	40 cc. 2N NaOH	4·15 (21° C.)	$7·1 \times 10^{-5}$	4·10	6·44	8·41	10·37	12·33	17·37	21·02	—	—	0·70	1·82	4·06	6·86	8·55

of this neutral protein solution were mixed with 2640 cc. water and 800 cc. N/1 HCl and the mixture put in an incubator (38°), and after 24 hours 30 cc. 5N HCl were added: 4 hours later 50 cc. 5N HCl were added and the mixture then kept another 24 hours at 38°.

(2) *Pepsin solution.* 10 g. scale preparation of pepsin was dissolved in 1000 cc. of 0·01N HCl; 10 cc. of this solution contained 1·6 mg. nitrogen.



For each experiment, as the table shows, 360 cc. acid albumin solution, 40 cc. of various mixtures of sodium hydrate and salt solution were mixed, and after warming to the temperature required there were added 100 cc. pepsin solution. The various solutions contained always the same salt concentration (about 0·16N NaCl). The digestion was carried out in an incubator at 38° C.

Precipitation with tin chloride or tannic acid solution was carried out in the manner of Sörensen. The hydrogen ion concentration of each test was electrometrically measured. It was found that the change of this concentration during the course of digestion was quite small and could be neglected.

Controls were made by heating the pepsin in a water bath to 100° for one hour, so that the ferment was thoroughly destroyed. From these experiments it was found that the nitrogen not precipitated by tannic acid or tin chloride solution in the control was constant during the whole digestion. 30 cc. of control solutions contained at the beginning as well as at the end of digestion 0·98 mg. nitrogen not precipitated by tannic acid, and 1·68 mg. not precipitated by tin chloride, so that these values were subtracted from the values obtained in the experiments with the active enzyme.

The results are shown in the accompanying table and curves.

From the table it is to be seen that the increase in the amount of nitrogen not precipitable with tin chloride is greatest in Exp. 5, $[H^{\cdot}] = 1{\cdot}7 \times 10^{-2}$, then follow Exps. 4, $[H^{\cdot}] = 4{\cdot}3 \times 10^{-2}$, and 3, $[H^{\cdot}] = 5{\cdot}8 \times 10^{-2}$, showing no considerable difference between them. The increase of that not precipitable by tannic acid is most obvious in Exp. 4, then come Exps. 3 and 5. Therefore the optimum seems to be at about Exp. 4, or between Exps. 4 and 5.

CONCLUSION.

The optimal point for the action of pepsin is about $[H^{\cdot}] = 4 \times 10^{-2}$. Between $[H^{\cdot}] = 5{\cdot}8 \times 10^{-2}$ and $1{\cdot}7 \times 10^{-2}$ there is no considerable difference in the rate of hydrolysis.

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XIII. ON THE OPTIMAL CONDITIONS FOR THE PROTEOCLASTIC ACTION OF TAKA-DIASTASE.

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Introduction. Taka-diastase is prepared from koji-yeast and is chiefly used for the artificial digestion of amylaceous food. Koji-yeast itself is produced by the influence of *Aspergillus oryzae* on soaked rice. *A. oryzae* produces various kinds of ferments. Atkinson [1881] was the first to note that koji contains a strong diastase. Kellner, Mori and Nagaoka [1890] found that it converts starch into maltose and dextrin and, finally, into grape sugar; moreover, that it converts cane sugar into invert sugar. Saito [1906] reported that *A. oryzae* produces also trypsin, rennet, and catalase. Wohlgemuth [1912] found also erepsin, lipase and a haemolytic ferment. The conditions of the diastatic action of koji-yeast or taka-diastase have been studied by various authors. Kellner, Mori and Nagaoka observed that lactic acid above 0·1 % weakens the action of koji-diastase, but in a concentration of 0·05 % accelerates its action somewhat. They found also that it is strongly influenced in its diastatic or inverting action by the presence of salt, but does not completely lose its action even with 20 % of salt content. Saito also found this influence of salt in the case of pure cultures of the organism. According to Leo [1896], taka-diastase is not so sensitive to acids as is ptyalin, while Strauss and Stargard [1898] found no difference between them. Wohlgemuth confirmed Leo and even found the addition of dilute hydrochloric acid to accelerate the action of taka-diastase. With regard to its proteoclastic function, Wohlgemuth found on the contrary that taka-diastase is more active in media which are neutral or very slightly alkaline than in those which are very slightly acid.

The clinical use of taka-diastase was studied by Leo, and by Strauss and Stargard. These authors found that its amyloclastic function is alone of importance. So far as my experience goes, taka-diastase is very effective also in the digestion of proteins. This fact is not surprising, since Wohlgemuth found that 1 g. taka-diastase contains as much proteoclastic ferment as 100 cc. of human or animal pancreatic juice. This proteoclastic function seems more important than the amyloclastic and is of more clinical value, since the digestion of proteins is more frequently defective than that of carbohydrates. Consequently it is desirable to study the optimal conditions in which this ferment acts. As the proteoclastic ferment contained in taka-diastase is very similar in its action to that of pancreatic juice, I applied the method which Michaelis and Davidsohn [1911] used to study trypsin, with some modification in details. As ferment I used commercial taka-diastase which was always freshly dissolved to 10 % concentration. This solution, if kept 24 hours or longer, becomes black in colour, so that titration is interfered with, though it does not lose its activity. As substrate Witte's peptone was used, dissolved to 4 % in distilled water by boiling. It was filtered and kept sterile. The conversion was determined by Sörensen's formal method [1908]. For each experiment there were taken 10 cc. of regulator solution mixed with 30 cc. of 4 % peptone solution and, after warming to the required temperature, 10 cc. of 10 % taka-diastase solution were added. 10 cc. of digestion mixture were taken immediately after mixing and afterwards at any desired time, were put into a beaker containing 10 cc. of 40 % formal to stop digestion and then titrated with N/10 NaOH. Ten drops of 1 % phenolphthalein solution were used and a difference of less than 0·1 cc. could be detected, as Michaelis and Davidsohn observed with trypsin. The temperature was kept between 37–38° and several comparative estimations were made at the same time to control each other. The reaction of the digestion mixtures was measured by the electrometric method, which was carried out at the temperature of the room, mentioned in each case. Following the method employed by Michaelis and Davidsohn in their research on trypsin, I added such a quantity of regulators (mostly phosphate or acetate mixtures) as to neutralise the influence of the products of digestion on the reaction. Even in 24 hours no noticeable change occurred. The results are given in Table I.

From Table I it is to be seen that the optimum for the action of taka-diastase on Witte's peptone is at $[H^{\cdot}] = 8{\cdot}5 \times 10^{-6}$. This is shown also graphically in the figure.

With regard to the resistance of taka-diastase to acids Strauss and Stargard

TABLE I.

No.	Regulator mixture	$[H^{\cdot}]$	P_H	Time mins.	N/10 NaOH formal titration cc.
1	5·0 cc. N/3 Na_2HPO_4 ...			20	0·10
	3·0 cc. N/1 NaOH ...	$9{\cdot}1 \times 10^{-12}$	11·04	40	0·28
	2·0 cc. distilled water ...		(19° C.)	60	0·39
2	5·0 cc. N/3 Na_2HPO_4 ...			20	0·94
	1·5 cc. N/1 NaOH ...	$8{\cdot}5 \times 10^{-9}$	8·07	40	1·74
	3·5 cc. distilled water ...		(17·5° C.)	60	2·34
3	5·0 cc. N/3 Na_2HPO_4 ...			20	1·08
	1·0 cc. N/1 NaOH ...	$8{\cdot}9 \times 10^{-9}$	8·05	40	1·80
	4·0 cc. distilled water ...		(19° C.)	60	2·40
4	5·0 cc. N/3 Na_2HPO_4 ...			20	1·34
	0·5 cc. N/1 NaOH ...	$4{\cdot}2 \times 10^{-8}$	7·38	40	2·02
	4·5 cc. distilled water ...		(17·5° C.)	60	2·72
5	0·7 cc. N/3 NaH_2PO_4 ...			20	1·42
	7·0 cc. N/3 Na_2HPO_4 ...	$9{\cdot}1 \times 10^{-8}$	7·04	40	2·25
	2·3 cc. distilled water ...		(19° C.)	60	3·10
6	Same as 5, but with			20	0
	boiled ferment			40	0
				60	0
7	4·0 cc. N/3 NaH_2PO_4 ...			20	1·62
	4·0 cc. N/3 Na_2HPO_4 ...	$4{\cdot}0 \times 10^{-7}$	6·40	40	2·53
	2·0 cc. distilled water ...		(16·5° C.)	60	3·35
8	4·5 cc. N/3 NaH_2PO_4 ...			20	1·62
	4·0 cc. N/3 Na_2HPO_4 ...	$4{\cdot}1 \times 10^{-7}$	6·39	40	2·65
	1·5 cc. distilled water ...		(16·5° C.)	60	3·40
9	7·0 cc. N/3 NaH_2PO_4 ...			20	1·67
	3·0 cc. distilled water ...	$1{\cdot}1 \times 10^{-6}$	5·96	40	2·65
			(17·5° C.)	60	3·40
10	1·0 cc. N/1 acetic acid ...			20	1·70
	3·0 cc. N/1 sodium acetate	$2{\cdot}1 \times 10^{-6}$	5·68	40	2·70
	6·0 cc. distilled water ...		(19·0° C.)	60	3·50
11	1·5 cc. N/1 acetic acid ...			20	1·75
	2·5 cc. N/1 sodium acetate	$4{\cdot}3 \times 10^{-6}$	5·37	40	2·80
	6·0 cc. distilled water ...		(17·0° C.)	60	3·60
12	2·0 cc. N/1 acetic acid ...			20	1·73
	2·0 cc. N/1 sodium acetate	$8{\cdot}5 \times 10^{-6}$	5·07	40	2·90
	6·0 cc. distilled water ...		(17° C.)	60	3·65
13	Same as 12			20	1·78
				40	2·90
				60	3·67
14	Same as 12			20	1·75
				40	2·85
				60	3·65
15	Same as 12			20	1·84
				40	2·82
				60	3·62
16	Same as 12, but with			20	0
	boiled ferment			40	0
				60	0

TABLE I (*continued*).

No.	Regulator mixture	[H·]	P_H	Time mins.	N/10 NaOH formal titration cc.
17	2·5 cc. N/1 acetic acid ...	$1{\cdot}6 \times 10^{-5}$	4·80 (20° C.)	20	1·62
	1·5 cc. N/1 sodium acetate			40	2·56
	6·0 cc. distilled water ...			60	3·53
18	3·0 cc. N/1 acetic acid ...	$2{\cdot}6 \times 10^{-5}$	4·59 (16° C.)	20	1·50
	0·3 cc. N/1 sodium acetate			40	2·30
	6·7 cc. distilled water ...			60	2·86
19	5·0 cc. N/3 NaH_2PO_4 ...	$1{\cdot}9 \times 10^{-4}$	3·72 (15° C.)	20	0·95
	2·0 cc. N/1 HCl			40	1·61
	3·0 cc. distilled water ...			60	2·05
20	Same as 19, but with boiled ferment			20	0
				40	0
				60	0
21	5·0 cc. N/3 NaH_2PO_4 ...	$1{\cdot}4 \times 10^{-2}$	1·84 (17° C.)	20	0·19
	5·0 cc. N/1 HCl			40	0·24
				60	0·29

found that the enzyme after one hour's contact with hydrochloric acid or gastric juice, of which the acidity is over 30 (0·11 %), loses entirely its amyloclastic power, so that no regeneration of its action in the intestine is to be expected when the medium resumes its neutral reaction. In respect to its proteoclastic function, my results are as follows:

Exp. 1. 1 g. taka-diastase was dissolved in 5·0 cc. N/10 HCl + 5·0 cc. N/10 NaOH. As regulator mixture was used 2·0 cc. acetic acid + 2·0 cc. sodium acetate + 6·0 cc. distilled water. Digestion was carried out in the way mentioned above.

Time mins.	Conversion N/10 NaOH cc.
20	1·75
40	2·93
60	3·75

Exp. 2. 1 g. taka-diastase was dissolved in 5·0 cc. N/10 HCl (at 19°). After one hour it was neutralised with 5·0 cc. N/10 NaOH. Other conditions the same as in Exp. 1.

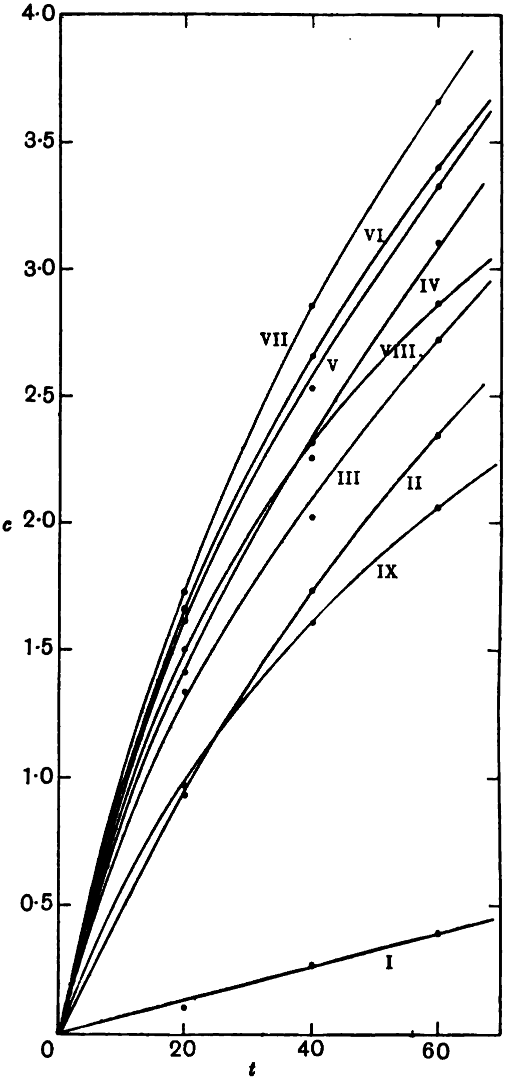
Time mins.	Conversion N/10 NaOH cc.
20	1·59
40	2·48
60	3·29

Exp. 3. Same as Exp. 2, only neutralised after two hours.

Time mins.	Conversion N/10 NaOH cc.
20	1·58
40	2·50
60	3·23

Exp. 4. 1 g. taka-diastase was dissolved in 5·0 cc. N/5 HCl + 5·0 cc. N/5 NaOH. Other conditions the same as in Exp. 1.

Time mins.	Conversion N/10 NaOH cc.
20	1·78
40	2·95
60	3·80



t = time in mins. *c* = conversion in cc.

Hydrogen ion concentrations.

I = $9{\cdot}1 \times 10^{-12}$ II = $8{\cdot}5 \times 10^{-9}$ III = $4{\cdot}2 \times 10^{-8}$ IV = $9{\cdot}1 \times 10^{-8}$ V = $4{\cdot}0 \times 10^{-7}$
VI = $1{\cdot}1 \times 10^{-6}$ VII = $8{\cdot}5 \times 10^{-6}$ VIII = $2{\cdot}6 \times 10^{-5}$ IX = $1{\cdot}9 \times 10^{-4}$

Exp. 5. 1 g. taka-diastase was dissolved in 5·0 cc. N/5 HCl. After one hour it was neutralised with 5·0 cc. N/5 NaOH. Other conditions the same as in Exp. 1.

Time mins.	Conversion N/10 NaOH cc.
20	0·73
40	1·33
60	1·60

Exp. 6. Same as Exp. 5, only neutralised after two hours.

Time mins.	Conversion N/10 NaOH cc.
20	0·70
40	1·22
60	1·55

Exp. 7. Same as Exp. 5, only neutralised after three hours.

Time mins.	Conversion N/10 NaOH cc.
20	0·65
40	1·10
60	1·45

From the above results one may judge that taka-diastase is not very sensitive to acid. During contact with hydrochloric acid below N/10 = 0·365 %, it loses its activity but little, on contact with N/5 HCl the diminution of its action is considerable; but this degree of acidity never happens in clinical cases. Longer or shorter duration of contact with acids, so far as relates to differences of one to three hours, has not so much influence as difference in degree of acidity. Hence, taka-diastase probably regains its action as soon as the medium resumes a suitable reaction. This justifies its clinical use.

As to the influence of salt on the proteoclastic function of taka-diastase, we see from the above results that 1 g. taka-diastase dissolved in 5 cc. N/10 HCl + 5 cc. N/10 NaOH or in 5 cc. N/5 HCl + 5 cc. N/5 NaOH has almost the same effect as that dissolved in 10 cc. distilled water, that is, taka-diastase dissolved in N/10 NaCl solution or in water has the same efficiency. Therefore the influence of salt in such concentrations is negligible.

Finally, it seems necessary to determine whether the results obtained apply to the tryptic or ereptic action. Wohlgemuth states that taka-diastase contains tryptic and ereptic ferments, like pancreatic juice; the action of the former is checked by blood serum while that of the other is accelerated by it. Witte's peptone [Schjerning, 1898] contains, besides albumose, a certain amount of albumin; so that in the experiments given above, these two ferments may both have been in action. The following table seems to prove this fact.

TABLE II.

No.	Regulator mixture	P_H	Time mins.	Conversion N/10 NaOH cc.
1	2·0 cc. acetic acid ...	5·07	20	1·75
	2·0 cc. sodium acetate		40	2·85
	6·0 cc. distilled water		60	3·65
2	2·0 cc. acetic acid ...	5·12	20	1·70
	2·0 cc. sodium acetate		40	2·75
	1·0 cc. ox serum ...		60	3·46
	5·0 cc. distilled water		—	—
3	2·0 cc. acetic acid ...	5·10	20	1·40
	2·0 cc. sodium acetate		40	2·60
	2·0 cc. dog serum ...		60	3·05
	4·0 cc. distilled water		—	—

By adding blood serum to the regulator mixture a slight diminution of digestion was observed, though no important change in the reaction was produced. Since the diminution of the action is of a slight character, whereas that of trypsin itself should be strongly checked by serum, it seems reasonable to hold that the method used applies mainly to the ereptic function, the tryptic one being subsidiary.

CONCLUSIONS.

1. The optimal reaction for the proteoclastic ferments contained in taka-diastase is at $[H^{\cdot}] = 8{\cdot}5 \times 10^{-6}$, *i.e.* they act better in a medium of slightly acid than in one of neutral or slightly alkaline nature.

2. These ferments are considerably resistant to acid; so that they regain their activity after neutralisation, if the acidity does not exceed the degree which usually obtains in the stomach of man or animals.

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XIV. THE ESTIMATION OF REDUCING SUGARS BY KENDALL'S SOLUTION AND THE CONSTRUCTION OF A TABLE INDICATING THE REDUCING POWER OF LAEVULOSE.

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While engaged in the estimation of sugars in plant tissues, we had occasion to employ Kendall's [1912] solution, consisting of copper sulphate, potassium carbonate, and salicylic acid. This was found by us to be extremely satisfactory and free from the possibility of error inherent in Fehling's solution, namely the solvent action of the hot sodium hydroxide upon the cuprous oxide. In all determinations Kendall's directions were carefully followed, but instead of estimating the copper reduced by this author's [1911] modification of the iodide method, or by the simpler routine adopted by Peters [1912], we proceeded in the following manner. The cuprous oxide was collected on asbestos in a Gooch crucible, washed with 360 cc. of hot distilled water (which quantity was found sufficient completely to remove the alkaline solution), then with a little alcohol and dried in a water oven. The crucible was placed inside a larger one and heated over a Teclu burner, as recommended by Davis and Daish [1913]. By heating in this manner for half an hour, all the cuprous oxide was converted into cupric oxide, and after allowing to cool in a desiccator for at least an hour, the crucible was weighed. The blue amphibole asbestos employed was treated with sodium hydroxide according to the well-known method of Munson and Walker.

By this method results were obtained for the reducing power of glucose, which were in close agreement with those in Kendall's table. Thus, 0·1345 g. of a sample of glucose, which had been recrystallised three times from 80 % alcohol and dried at 89°, gave 0·4745 g. of cupric oxide, the amount given in the table being 0·4743 g. This, it is worthy of note, was the first determination made by the authors by means of Kendall's solution.

Having thus satisfied ourselves that the method was reliable where pure sugars were concerned, it became necessary to ascertain whether the presence of citrates had any disturbing effect, since Davis and Daish [1913] have shown that citric acid inverts sucrose completely under conditions in which it does not hydrolyse maltose appreciably. Kendall himself tried to adapt Benedict's volumetric method to gravimetric sugar estimations, but came to the conclusion that the citrate in the solution employed, while dissolving but little cuprous oxide, had a depressing effect on the reducing power of the sugar. The following experiments show that it is not permissible to employ 2 % citric acid for the inversion of sucrose, when it is intended to estimate the products of hydrolysis by Kendall's method. Kahlbaum's sucrose from Indian cane was taken, the weight being corrected for the moisture present. The latter was determined by heating the finely powdered sugar in a closed vessel containing phosphoric oxide, the temperature being approximately 100°, till a constant weight was obtained.

A quantity of sucrose, 0·518 g., was made up to 100 cc. (solution *A*) with 2 g. of citric acid, and 20 cc. portions were heated for various times in a boiling water bath. Polarimetric readings showed that the change was not complete in fifteen minutes but was in thirty minutes.

For these observations and for the reductions the aliquot portions were made up to 25 cc. (solution *B*).

	CuO	
Solution *B*	20 min. heating	60 min. heating
20	0·3051 g.	0·3057 g.

From Kendall's table these values correspond to 0·495 g. sucrose per 100 cc. original solution. Thus it is clear that the citrate interferes seriously with the accuracy of the method. Accordingly it is advisable to employ invertase, free from maltase, for the inversion of sucrose in plant extracts since hydrochloric acid attacks laevulose and dextrose also to some extent.

Kendall's tables are constructed for glucose, invert sugar, lactose and maltose. As it was necessary for the purposes of our research to estimate laevulose with accuracy, a series of direct determinations of the reducing power of this sugar were made. In these experiments Kahlbaum's "laevulose from inulin I" was weighed out after prolonged drying in a desiccator. A portion of this very finely powdered was heated to the temperature of the water oven in a desiccating bottle connected with a tube containing phosphoric oxide, this tube being attached to an air-pump. After an hour of heating no loss of weight, whatever, was found to have occurred and the sugar was therefore taken to be dry. The powder remained snow white throughout. Taking $[\alpha]_D^{20^\circ} = -92{\cdot}5^\circ$ for laevulose, a solution was found to contain 2·4760 g. by calculation from the rotation measured in a 20 cm. tube at 16·6° and corrected to 20°. The polarimeter used gave readings to a hundredth of a degree. As 2·4851 g. of laevulose had been weighed out the agreement was considered satisfactory and the laevulose was shown to be of a high degree of purity. The weights of the laevulose and of the cupric oxide thus obtained in the reductions were employed in the calculation of an equation of the form $y = a + bx + cx^2$, by the method of least squares. In this equation y denotes the weight of oxide and x represents that of the laevulose, both in grams. It should be noted here that the constants in Kendall's equation are intended for calculations in milligrams.

Evaluation of the constants gave the following figures:

$$y = 0{\cdot}0006 + 3{\cdot}910x - 2{\cdot}601x^2.$$

The observed and calculated values of y are recorded below.

x	y calculated	y found	Difference
0·0321	0·1234	0·1230	+0·0004
0·0400	0·1528	0·1533	−0·0005
0·0493	0·1871	0·1856	+0·0015
0·0777	0·2887	0·2914	−0·0027
0·0926	0·3404	0·3406	−0·0002
0·1285	0·4601	0·4611	−0·0010

From these figures it is clear that the equation yields values which agree fairly well with those found by direct experiment.

The accuracy is increased by the large amount of oxide obtainable by Kendall's method relatively to the amount of sugar employed. Thus the ratio CuO/laevulose varies from 3·83 to 3·58 in the above table.

Accordingly an error of 0·001 g. cupric oxide corresponds to less than 0·0003 g. laevulose. In the table which follows, the weights of oxide were calculated for every two milligrams of laevulose and the intermediate values interpolated. It may be added that for some as yet obscure reason it is not advisable to estimate laevulose by this method beyond the limits of concentration recorded in our table. Very numerous determinations were carried out at higher concentrations but the results obtained were not of such a degree of accuracy as to justify their use in constructing a table. We hope to study the point further.

In conclusion we wish to acknowledge our indebtedness to Prof. H. H. Dixon for all facilities for carrying out this work in the School of Botany, Trinity College, Dublin; the expenses were defrayed by the funds of the laboratory.

Laevulose mg.	Cupric oxide mg.
10·0	39·4
10·4	40·9
10·8	42·5
11·2	44·0
11·6	45·6
12·0	47·1
12·4	48·6
12·8	50·2
13·2	51·7
13·6	53·3
14·0	54·8
14·4	56·3
14·8	57·9
15·2	59·4
15·6	61·0
16·0	62·5
16·4	64·0
16·8	65·6
17·2	67·1
17·6	68·7
18·0	70·2
18·4	71·7
18·8	73·2
19·2	74·8
19·6	76·3
20·0	77·8
20·4	79·3
20·8	80·8
21·2	82·4
21·6	83·9
22·0	85·4
22·4	86·9
22·8	88·4
23·2	89·9
23·6	91·4
24·0	92·9
24·4	94·4
24·8	95·9
25·2	97·4
25·6	98·9
26·0	100·4
26·4	101·9
26·8	103·5
27·2	105·0
27·6	106·6
28·0	108·1
28·4	109·6
28·8	111·1
29·2	112·6
29·6	114·1
30·0	115·6
30·4	117·1
30·8	118·5
31·2	120·0
31·6	121·5
32·0	123·0
32·4	124·5
32·8	126·0
33·2	127·5
33·6	129·0
34·0	130·5
34·4	132·0
34·8	133·5
35·2	135·0
35·6	136·5
36·0	138·0
36·4	139·5
36·8	140·9
37·2	142·4
37·6	143·9
38·0	145·4
38·4	146·9
38·8	148·3
39·2	149·8
39·6	151·3
40·0	152·8
40·4	154·3
40·8	155·8
41·2	157·3
41·6	158·8
42·0	160·3
42·4	161·8
42·8	163·2
43·2	164·7
43·6	166·2
44·0	167·7
44·4	169·1
44·8	170·6
45·2	172·0
45·6	173·5
46·0	175·0
46·4	176·4
46·8	177·9
47·2	179·3
47·6	180·8
48·0	182·3
48·4	183·7
48·8	185·2
49·2	186·6
49·6	188·1
50·0	189·6
50·4	191·0
50·8	192·5
51·2	193·9
51·6	195·4
52·0	196·9
52·4	198·3
52·8	199·8
53·2	201·2
53·6	202·7
54·0	204·2
54·4	205·6
54·8	207·1
55·2	208·5
55·6	210·0
56·0	211·4
56·4	212·8
56·8	214·3
57·2	215·7
57·6	217·2
58·0	218·7
58·4	220·1
58·8	221·5
59·2	222·9
59·6	224·4
60·0	225·8
60·4	227·2
60·8	228·7
61·2	230·1
61·6	231·6
62·0	233·0
62·4	234·4
62·8	235·9
63·2	237·3
63·6	238·8
64·0	240·2
64·4	241·6
64·8	243·0
65·2	244·4
65·6	245·9
66·0	247·3
66·4	248·7
66·8	250·2
67·2	251·6
67·6	253·1
68·0	254·5
68·4	255·9
68·8	257·3
69·2	258·7
69·6	260·2
70·0	261·6
70·4	263·0
70·8	264·4
71·2	265·8
71·6	267·2
72·0	268·6
72·4	270·0
72·8	271·5
73·2	272·9
73·6	274·4
74·0	275·8
74·4	277·2

Laevulose mg.	Cupric oxide mg.
74·8	278·6
75·2	280·0
75·6	281·4
76·0	282·8
76·4	284·2
76·8	285·6
77·2	287·0
77·6	288·4
78·0	289·8
78·4	291·2
78·8	292·6
79·2	294·0
79·6	295·4
80·0	296·8
80·4	298·2
80·8	299·5
81·2	300·9
81·6	302·3
82·0	303·7
82·4	305·1
82·8	306·5
83·2	307·9
83·6	309·4
84·0	310·8
84·4	312·2
84·8	313·5
85·2	314·9
85·6	316·3
86·0	317·6
86·4	319·0
86·8	320·4
87·2	321·8
87·6	323·3
88·0	324·7
88·4	326·0
88·8	327·4
89·2	328·7
89·6	330·1
90·0	331·4
90·4	332·8
90·8	334·1
91·2	335·5
91·6	336·9
92·0	338·3
92·4	339·7
92·8	341·0
93·2	342·4
93·6	343·8
94·0	345·2
94·4	346·6
94·8	347·9
95·2	349·3
95·6	350·7
96·0	352·0
96·4	353·4
96·8	354·7
97·2	356·1
97·6	357·5
98·0	358·8
98·4	360·2
98·8	361·5
99·2	362·9
99·6	364·3
100·0	365·6
100·4	366·9
100·8	368·3
101·2	369·6
101·6	370·9
102·0	372·3
102·4	373·7
102·8	375·0
103·2	376·4
103·6	377·8
104·0	379·1
104·4	380·5
104·8	381·8
105·2	383·2
105·6	384·6
106·0	385·9
106·4	387·2
106·8	388·5
107·2	389·9
107·6	391·2
108·0	392·6
108·4	393·9
108·8	395·2
109·2	396·5
109·6	397·9
110·0	399·2
110·4	400·5
110·8	401·8
111·2	403·2
111·6	404·5
112·0	405·9
112·4	407·2
112·8	408·5
113·2	409·9
113·6	411·2
114·0	412·6
114·4	413·9
114·8	415·2
115·2	416·5
115·6	417·9
116·0	419·2
116·4	420·5
116·8	421·8
117·2	423·1
117·6	424·5
118·0	425·8
118·4	427·1
118·8	428·4
119·2	429·7
119·6	431·0
120·0	432·3
120·4	433·6
120·8	434·9
121·2	436·3
121·6	437·6
122·0	439·0
122·4	440·3
122·8	441·6
123·2	442·9
123·6	444·2
124·0	445·5
124·4	446·8
124·8	448·1
125·2	449·4
125·6	450·7
126·0	452·0
126·4	453·3
126·8	454·6
127·2	455·9
127·6	457·2
128·0	458·5
128·4	459·8
128·8	461·1
129·2	462·4
129·6	463·7
130·0	465·0

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XV. THE GALACTOSIDES OF THE BRAIN. IV. THE CONSTITUTION OF PHRENOSIN AND KERASIN.

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(*Received February 23rd, 1916.*)

In the first communication of this series [1913, 2] I expressed the view that the brain contains in a preformed condition mainly two representatives of the group of glucosides, namely the galactosides phrenosin and kerasin, which were originally discovered by Thudichum. A new method for their preparation was subsequently described together with a physical test (the selenite plate test) which furnishes a reliable method for controlling the efficacy of the methods used for their separation [1914, 1].

In the following communication are given the experimental data obtained by the chemical and physical examination of the galactosides together with the results of complete hydrolysis experiments.

The outcome of these experiments has been to establish for the first time the fact that kerasin possesses a laevorotation in distinction from the dextrorotatory phrenosin, and further that the only difference in chemical constitution of the two glucosides consists in their fatty acid component[1].

Whilst kerasin furnishes on hydrolysis inactive lignoceric acid ($C_{24}H_{48}O_2$), phrenosin gives rise to the optically active hydroxy-acid phrenosinic acid ($C_{25}H_{50}O_3$). The near relationship of these fatty acids is evident from the interesting fact that phrenosinic acid furnishes lignoceric acid on oxidation [Levene and Jacobs, 1912, 2]. Both glucosides yield the same carbohydrate, *d*-galactose, and the same base, sphingosine ($C_{17}H_{35}O_2N$), on hydrolysis.

These observations, together with the facts established by Levene and others with regard to the constitution of sphingosine, enable us now to construct constitutional formulae for these substances.

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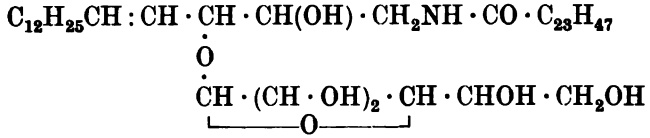
[1] The results of this work were communicated to the section of physiology of the 17th Internat. Congress of Medicine on Aug. 11th, 1913 [Rosenheim, 1913, 1]. Their publication in full has been delayed owing to unforeseen circumstances.

The unsaturated base sphingosine contains two free hydroxyl groups besides one amino-group [Levene and Jacobs, 1912, 1; Thierfelder and Thomas, 1912; Levene and West, 1914], and its formula may be written as: $C_{12}H_{25}CH:CH\cdot CH(OH)\cdot CH(OH)\cdot CH_2NH_2$. It is evident that in the galactosides the amino-group of the sphingosine is no longer free, since kerasin does not yield any nitrogen with nitrous acid when tested in van Slyke's apparatus. I was also unable to obtain any nitrogen from phrenosin by heating with hypobromite of potassium, and Levene and Jacobs [1912, 3] record incidentally a similar experience when testing their cerebrins in van Slyke's apparatus. We may therefore assume that in the galactosides the NH_2 group of sphingosine is in combination with the carboxylic group of the fatty acids, especially since the substances show neither any acid nor alkaline tendencies[1]. The possibility of a linkage of sphingosine by means of the NH_2 group with galactose must not be lost sight of. Up till now, however, no naturally occurring glucosides have been met with in which the carbohydrate is linked to nitrogen, and it is therefore much more likely that the aldehyde group of the galactose enters into combination with one of the hydroxyl groups of sphingosine. In analogy to the constitution of the other known naturally occurring glucosides, we may further conclude that phrenosin and kerasin are β-galactosides, in which galactose possesses the closed chain formation. From these considerations we arrive at the following constitutional formula of kerasin ($C_{47}H_{91}O_8N$):



$$\begin{array}{l}C_{12}H_{25}CH:CH\cdot CH\cdot CH(OH)\cdot CH_2NH\cdot CO\cdot C_{23}H_{47}\\ \qquad\qquad\qquad\quad\;\, O\\ \qquad\qquad\qquad\quad\;\, \underbrace{CH\cdot (CH\cdot OH)_2\cdot CH}_{O}\cdot CHOH\cdot CH_2OH\end{array}$$

This formula is in agreement with all the known facts of the reactions and cleavage products of kerasin. It allows for the formation of galactose, lignoceric acid and a salt of sphingosine during hydrolysis with a mineral acid in aqueous solution, whilst it also explains the formation of mono-methyl sphingosine and the methyl ester of lignoceric acid during methyl alcoholysis (see later). It contains five free hydroxyl groups which is in accordance with the formation of a penta-acetyl kerasin, a derivative prepared by Thierfelder [1914]. The position of the unoccupied hydroxyl group in the sphingosine residue in the above formula is arbitrary, since the relative position of the hydroxyl groups in sphingosine itself is still unknown.

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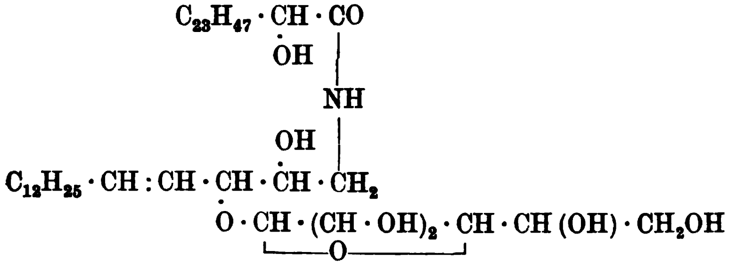
[1] The tentatively advanced formulae [Rosenheim, 1913, 1] in which the presence of a free NH_2 group is assumed require therefore a modification in this direction.

The constitution of phrenosin is analogous to that of kerasin, the hydroxy-acid phrenosinic acid ($C_{25}H_{50}O_3$) taking the place of lignoceric acid. This is expressed by the following constitutional formula of phrenosin ($C_{48}H_{93}O_9N$):



$$\begin{array}{l}
\qquad\qquad\qquad C_{23}H_{47}\cdot \underset{OH}{CH}\cdot CO \\
\qquad\qquad\qquad\qquad\qquad\quad | \\
\qquad\qquad\qquad\qquad\qquad\quad NH \\
\qquad\qquad\qquad\qquad\qquad\quad | \\
C_{12}H_{25}\cdot CH:CH\cdot CH\cdot \overset{OH}{CH}\cdot CH_2 \\
\qquad\qquad\qquad\quad | \\
\qquad\qquad\qquad\quad O\cdot \underbrace{CH\cdot (CH\cdot OH)_2\cdot CH}_{O}\cdot CH(OH)\cdot CH_2OH
\end{array}$$

We must assume that in the phrenosin molecule the two hydroxyl groups of sphingosine are at all events partially combined with other groups, since on alcoholysis besides sphingosine a certain amount of dialkyl sphingosine is formed. It is most probable that partial anhydride formation takes place between the sphingosine and phrenosinic acid hydroxyls. It is not likely that one of the hydroxyl groups of the galactose molecule is thus linked up, since on this assumption one would expect to find at least an indication of the formation of an alkyl galactose on alcoholysis. The linkage between the hydroxyl groups of phrenosinic acid and sphingosine is evidently only partial, since phrenosin furnishes on acetylation a hexa-acetyl derivative [Thierfelder, 1914]. This assumption also allows a satisfactory explanation for the existence of the two modifications of phrenosin. The ordinary "amorphous" phrenosin, which easily assumes the liquid-crystalline form [Rosenheim, 1914, 2], corresponds to the anhydric formula, whilst in the crystallised phrenosin the two hydroxyl groups are free. In accordance with this view is the fact that the crystallised modification contains one molecule of water more than the amorphous modification [Rosenheim, 1914, 2; see also Thierfelder and Wörner, 1900]. If this view is correct, the completely crystallised modification should give rise to a monoalkyl sphingosine on alcoholysis, a possibility which I have not yet tested experimentally. It may, however, be noted in this direction that Thierfelder and Thomas [1912] obtained on methyl alcoholysis of a sample of "cerebron" a substance which they considered at first as a monomethyl sphingosine. As the "cerebron" employed in this experiment consisted of a mixture of the crystallised and amorphous modifications, it is not unlikely that the cleavage product was a mixture of sphingosine with monomethyl sphingosine.

It is to be expected that the study of the products of a partial hydrolysis of these galactosides will furnish conclusive evidence to decide the correctness of the constitutional formulae advanced above, which may serve in any case as a basis for further research. Thudichum has already indicated the existence of two such compounds, which he called aesthesin (phrenosin minus galactose) and psychosin (phrenosin minus phrenosinic acid). The author is at present engaged in studying the action of enzymes on these galactosides. It is hoped that a suitable lipoclastic enzyme may split off the fatty acid component, leaving a sphingosine-galactoside, whilst an enzyme of the emulsin type may split off galactose and leave the sphingosine-fatty acid combination intact. The experiments made up till now show that phrenosin is resistant to pancreatic lipase and emulsin (commercial). An extract of brain from a case of extensive brain softening also had no action on phrenosin.

EXPERIMENTAL.

I. *Phrenosin.*

Elementary analysis. The analyses (1) and (2) refer to different samples of amorphous phrenosin prepared by the new pyridine method [Rosenheim, 1914, 1]. Analysis (3) was made of a sample of crystallised phrenosin [Rosenheim, 1914, 2]. The substances were dried at 105°.

(1) 0·1244 g.; 0·3183 g. CO_2; 0·1293 g. H_2O,
0·1293 g.; 2·3 cc. N at 26·0° and 714·5 mm.
(2) 0·1255 g.; 0·3207 g. CO_2; 0·1267 g. H_2O,
0·1321 g.; 2·2 cc. N at 26·0° and 714·5 mm.
(3) 0·1300 g.; 0·3305 g. CO_2; 0·1291 g. H_2O,
0·0960 g.; 1·7 cc. N at 17·5° and 722·5 mm.

	(1)	(2)	(3)	Average	Calc. for $C_{48}H_{93}O_9N$
C	69·78 %	69·69 %	69·34 %	69·60 %	69·65 %
H	11·63 ,,	11·29 ,,	11·11 ,,	11·34 ,,	11·24 ,,
N	1·90 ,,	1·79 ,,	1·91 ,,	1·83 ,,	1·70 ,,

Melting point. Phrenosin on heating assumes the liquid-crystalline condition, from a temperature of about 95° upwards until 210–212°, when it changes into the amorphous liquid condition. These phenomena have been fully described in a previous communication [1914, 2]. Phrenosin prepared by Thudichum's original method, as well as by that of Thierfelder, shows the same behaviour as phrenosin prepared by the pyridine method.

Optical activity. Phrenosin is dextrorotatory and the degree of its rotation is greatly influenced by the nature of the solvent, temperature and concentration. Pyridine is the most convenient solvent at ordinary temperatures and was first used in 1907 for this purpose by Rosenheim and Tebb in connection with some other work on brain lipoids. A 10 % solution of phrenosin in pyridine, however, possesses at 20° only about half the optical activity of a 5 % solution in chloroform-methyl alcohol at 40°, whilst a 10 % solution in the latter solvent at 45° shows nearly three times the optical activity of a 10 % pyridine solution at 20°. The estimations were made in a large Lippich polarimeter in a 1 dm. tube in sodium light.

Phrenosin (Pyridine method).

(1) 1·0046 g. in 10 cc. pyridine; $t = 20°$; $a = +0·38°$; $[a]_D^{20°} = +3·78°$.

(2) 1·0011 g. in 10 cc. pyridine; $t = 20°$; $a = +0·37°$; $[a]_D^{20°} = +3·70°$.

1·0000 g. in 10 cc. chloroform-methyl alcohol (3 : 1); $t = 45°$; $a = +1·04°$; $[a]_D^{45°} = +10·4°$.

Phrenosin (Thudichum's method).

1·000 g. in 10 cc. pyridine; $t = 18°$; $a = +0·37°$; $[a]_D^{18°} = +3·70°$.

1·000 g. in 10 cc. pyridine; $t = 30°$; $a = +0·43°$; $[a]_D^{30°} = +4·30°$.

0·500 g. in 10 cc. chloroform-methyl alcohol (3 : 1); $t = 40°$; $a = +0·37°$; $[a]_D^{40°} = +7·40°$.

Thierfelder and Kitagawa [1906] have previously found for "cerebron" $[a]_D^{50°} = +6·4$ to $+8·4°$ in 5 % chloroform-methyl alcohol and Levene and Jacobs [1912, 3] for "*d*-cerebrin" (prepared by the baryta method) $[a]_D^{25°} = +1·01$ to $+1·88°$ in pyridine. Later Levene [1913] has examined "cerebrin" fractions obtained by a process not yet fully described, for which he found $[a]_D = +3·05$ to $+4·14°$ in pyridine and $[a]_D = +9·5$ to $+10·7°$ in 6 % chloroform-methyl alcohol. My results agree, therefore, both with the earlier ones of Thierfelder and the later ones of Levene.

Hydrolysis. Many samples of phrenosin, prepared both by Thudichum's original method and by the pyridine method were hydrolysed. The products of hydrolysis were identical in both cases, qualitatively as well as quantitatively, namely (1) phrenosinic acid, (2) sphingosine and (3) galactose. Hydrolysis was effected by boiling under ordinary pressure (*a*) with dilute aqueous hydrochloric acid, or (*b*) with methyl alcoholic sulphuric acid. In the latter case the methyl-ester of phrenosinic acid and small quantities of dimethylsphingosine, in addition to sphingosine, are formed.

(1) *Phrenosinic acid* ($C_{25}H_{50}O_3$)[1]. 1·0 g. phrenosin (prepared by the

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[1] Phrenosinic acid = Thudichum's "neurostearic" acid = Thierfelder's "cerebronic" acid. (See O. Rosenheim, 1913, 2.)

pyridine method) was hydrolysed by boiling its solution in 50 cc. methyl alcohol, containing 5 % sulphuric acid, for 5·5 hours on a reflux. The solution remained perfectly colourless and on cooling a crystallised white precipitate formed. The mixture of free fatty acid and ester was filtered off, washed with cold methyl alcohol and converted into the sodium salt by means of sodium methylate in methyl alcoholic solution. The sodium salt was recrystallised from boiling absolute alcohol and the acid liberated by dilute sulphuric acid. The free acid was taken up in ether and its ethereal solution washed with water in a separating funnel until free from mineral acid. The white fatty acid left on evaporation of the filtered ether solution weighed 0·305 g. and fused at 103°–104°.

Phrenosinic acid crystallises from alcohol in characteristic forms which Thudichum [1881] has described as "mamillary" or cauliflower-like masses. Under the polarising microscope they are seen to consist of a conglomerate of irregular spherocrystals, which behave in the selenite plate test [Rosenheim, 1914, 1] exactly as the mother substance phrenosin.

For analysis the acid was recrystallised three times from acetone. After the last recrystallisation and drying *in vacuo* it showed a melting point of 105°–106° and a dextrorotation of $[\alpha]_D^{20°} = +3{\cdot}86°$ in pyridine ($l = 1$; $c = 2{\cdot}849$; $t = 20°$; $\alpha = +0{\cdot}11°$).

On analysis it gave the following figures:

0·0792 g.; 0·2195 g. CO_2; 0·0894 g. H_2O.

	Found	Calc. for $C_{25}H_{50}O_3$
C	75·58 %	75·30 %
H	12·63 „	12·65 „

Sodium salt. Another preparation of phrenosinic acid was converted into the sodium salt, which was recrystallised from absolute alcohol and dried at 105°. 0·1042 g. gave 0·0172 g. Na_2SO_4;

Na = 5·39 %. Calculated for $C_{25}H_{49}O_3Na$ = 5·48 %.

Molecular weight estimation. A sample of phrenosin, prepared by Thudichum's method, was hydrolysed as above described. The ester was converted into the potassium salt and the free acid, prepared from it, recrystallised from acetone. It fused at 102°–103°. The acid was dissolved in benzene and titrated with 0·1N alcoholic potash (phenolphthalein as indicator). 0·3257 g. required 8·24 cc. 0·1N KOH.

Molecular weight = 395. Calc. for $C_{25}H_{50}O_3$: 398.

Another sample was hydrolysed by boiling for 24 hours with dilute hydrochloric acid. The mixture of free fatty acid and sphingosine hydrochloride was filtered after the addition of a few cc. of saturated sodium sulphate solution which renders quick filtration possible. The acid was extracted with ether and purified by means of the barium salt. The barium salt was decomposed with tartaric acid and the free acid extracted with ether. It fused at 102°–103°. 0·2687 g. required 6·73 cc. 0·1N KOH. Molecular weight = 399. Calc. for $C_{25}H_{50}O_3$: 398.

Melting point of phrenosinic acid. The fatty acid obtained by Thudichum from phrenosin and called by him "neurostearic" acid had a melting point of 84°, but the same elementary composition as phrenosinic acid. Thierfelder's "cerebronic" acid, prepared by a method almost identical with that of Thudichum, melted at 98°–99° but yielded on analysis practically the same figures as Thudichum's acid. Mainly on account of the difference in their melting points, Thierfelder [1905] declined to admit the identity of these acids and their mother substances. More recently, however, Levene and Jacobs [1912, 2] again obtained Thudichum's acid of the lower melting point and showed that "cerebronic" acid exists in two optically isomeric forms of which the inactive one melts at 82°–85° (Thudichum's "neurostearic" acid), whilst the dextrorotatory form melts at 106°–108° (Thierfelder's "cerebronic" acid). A mixture of the two forms melting at 92°–95° is frequently obtained [Rosenheim and MacLean, 1915]. The conditions under which these two isomers are formed are not yet explained. In some preliminary experiments I have found that the melting point of the dextrorotatory acid (103°–104°) is lowered considerably by keeping it for some time in a toluene bath. After six hours the melting point had fallen to 88°–89°. Experiments are in progress to decide whether this decrease goes hand in hand with a racemisation. The change in melting point may possibly be due also to the formation of an anhydride or lactone-like derivative owing to the presence of a hydroxyl group in phrenosinic acid. The difficulty mentioned by Levene and West [1913] of obtaining correct titration values of the inactive acid is in favour of such an explanation.

(2) *Sphingosine* ($C_{17}H_{35}O_2N$). This base was first obtained by Thudichum on hydrolysis of phrenosin with dilute sulphuric acid under pressure. The peculiar name was given to it "in commemoration of the many enigmas which it presented to the enquirer" [Thudichum, 1881]. Thierfelder [1904] confirmed Thudichum's results when he hydrolysed his "cerebron" by the same method; but later Thierfelder and Kitagawa [1906] expressed doubts

as to the uniformity of sphingosine, as they obtained on hydrolysis in methyl alcoholic solution a mixture of sphingosine with another base of the composition $C_{19}H_{39}O_2N$. These doubts were removed by Levene and Jacobs [1912, 1], who showed that the second base, dimethylsphingosine, was formed during alcoholysis and was not originally present in the phrenosin molecule. These observations were confirmed by Thierfelder and Riesser [1912].

I have obtained sphingosine from phrenosin (prepared by Thudichum's method) on hydrolysis with dilute hydrochloric acid, and both sphingosine and dimethylsphingosine from phrenosin (prepared by the pyridine method) on hydrolysis with sulphuric acid in methyl alcoholic solution. Only the identification of the latter need be described here.

The filtrate obtained above after the removal of phrenosinic acid and its ester was diluted with 100 cc. water and the alcohol evaporated on a water-bath. The solution became cloudy and a semi-solid white substance collected on the surface, which solidified on cooling. This was filtered off and dissolved on the filter in boiling alcohol. On evaporation of the alcohol and drying *in vacuo* the residue of sphingosine sulphate and dimethylsphingosine sulphate weighed 0·550 g. The two substances were separated by their different solubilities in alcohol.

(*a*) *Sphingosine.* The mixture of the sulphates was treated repeatedly at room temperature with alcohol. From the insoluble portion the base was set free by means of alcoholic potash. It was extracted with ether and the ether solution washed with water. On evaporation of the ether the base remained as a syrup, which crystallised completely in rosettes of needles. The base was dissolved in alcohol and reconverted into the sulphate by the addition of alcoholic sulphuric acid. The process of purification was repeated once more and sphingosine sulphate was obtained in perfectly white crystals, which were filtered, washed with ether and dried *in vacuo*. The salt is hygroscopic and melts with decomposition at 233°–235° (Levene and Jacobs give 233°–239°). Its solution in methyl alcoholic sulphuric acid showed a laevorotation of $[\alpha]_D^{18^\circ} = -9{\cdot}47^\circ$.

$$(l = 1;\quad c = 1{\cdot}585;\quad t = 18^\circ;\quad \alpha = -0{\cdot}15^\circ.)$$

(*b*) *Dimethylsphingosine.* The easily soluble sulphate of dimethylsphingosine obtained above was converted into the free base and extracted with ether. The base crystallised at once on evaporation of the ether. It was converted into the hydrochloride by adding a methyl alcoholic solution of hydrochloric acid to its solution in methyl alcohol. The hydrochloride was recrystallised from acetone, from which it separated in glistening plates. It melted at 133°–134° and gave the following results on analysis:

5·385 mg.; 12·875 mg. CO_2; 5·84 mg. H_2O.

	Found	Calc. for $C_{19}H_{39}O_2N \cdot HCl$
C	65·20 %	65·18 %
H	11·58 ,,	11·52 ,,

(3) *d-Galactose.* The solution from which phrenosinic acid and sphingosine had been removed, as above described, contained only *d*-galactose as the last product of hydrolysis. This was estimated quantitatively by means of the polarimeter. The solution was made up to 100 cc. and showed in a 2 dm. tube a rotation of $a = + 0·35°$. Whence galactose = 21·63 %; calc. for 1 mol. galactose from $C_{48}H_{93}O_9N$21·77: %.

d-Galactose was identified by means of its characteristic methylphenylhydrazone. Solid sodium acetate was added until the reaction was no longer acid to Congo paper. A few drops of methylphenylhydrazine were added, and after standing at room temperature crystals of the hydrazone settled down. They were filtered off, washed with water and recrystallised from alcohol. The perfectly white crystals of the hydrazone melted sharply at 190°. Neuberg [1907] gives the melting point of galactose methylphenylhydrazone at 191°.

Comparison of the galactoside, prepared from brain by the pyridine method, with Thudichum's phrenosin shows their complete agreement in all points. It follows from this that Thierfelder's "cerebron," with which my substance agrees in every way, is also identical with phrenosin. Gamgee's "pseudo-cerebrin" is admittedly identical with "cerebron" prepared by Thierfelder's original method (1904) whilst there can be no doubt that the "cerebrin" of Parcus as well as that of Kossel and Freytag is identical with "cerebron" prepared by Thierfelder's later baryta method [1913].

Hence it follows that all these products represent one and the same substance, namely phrenosin. The unfortunate confusion which has been brought into the nomenclature of the brain lipoids by the introduction of new names for one and the same substance can now be considered to be cleared away experimentally. It seems only just to retain the name phrenosin introduced in 1874 by Thudichum, who discovered the substance and was the first to establish the nature of its cleavage products. This suggestion has previously been made, based on theoretical considerations, by Posner and Gies [1906] and Rosenheim and Tebb [1907] and was adopted by myself in the proposals for the nomenclature of the lipoids [1909].

II. *Kerasin.*

Elementary analysis. The analyses (1)–(4) were made of different samples of kerasin prepared by the new pyridine method and sample (5) refers to kerasin prepared by Thudichum's original method, slightly modified [Rosenheim, 1914, 1]. The substances were dried *in vacuo* over sulphuric acid.

(1) 0·1363 g.; 0·3444 g. CO_2; 0·1395 g. H_2O,
0·1416 g.; 2·4 cc. N at 24° C. and 714 mm.
(2) 0·1317 g.; 0·3331 g. CO_2; 0·1372 g. H_2O.
(3) 0·1200 g.; 0·3034 g. CO_2; 0·1205 g. H_2O,
0·1379 g.; 2·3 cc. N at 25° C. and 714·5 mm
(4) 0·1185 g.; 0·2997 g. CO_2; 0·1187 g. H_2O,
0·1285 g.; 2·2 cc. N at 21° C. and 716 mm.
(5) 0·1156 g.; 0·2920 g. CO_2; 0·1184 g. H_2O,
0·1578 g.; 2·6 cc. N at 22·5° C. and 710 mm.

	(1)	(2)	(3)	(4)	(5)	Calc. for $C_{47}H_{91}O_8N+H_2O$
C	68·91	68·98	68·95	68·98	68·90	69·14 %
H	11·44	11·65	11·23	11·21	11·46	11·49 ,,
N	1·83	—	1·74	1·86	1·76	1·72 ,,

The values obtained are somewhat lower than those previously recorded in the literature. This may be due to the fact that the substances were dried *in vacuo* at ordinary temperature instead of at 100°–105°. The figures agree well with the theoretical formula $C_{47}H_{91}O_8N + H_2O$ which is calculated on the assumption that the kerasin molecule is composed of lignoceric acid ($C_{24}H_{48}O_2$), sphingosine ($C_{17}H_{35}O_2N$) and galactose ($C_6H_{12}O_6$) in equimolecular proportions.

It may be pointed out again that the elementary analysis is an unreliable index of the purity of these complex galactosides and does not prove the absence of phrenosin in kerasin preparations and *vice versa.* The difference in elementary composition between phrenosin ($C_{48}H_{93}O_9N$) and kerasin ($C_{47}H_{91}O_8N$) is so small that the presence of even a large percentage of phrenosin cannot be detected by elementary analysis. This is well illustrated by the experience of Levene and Jacobs [1912, 3], who originally considered phrenosin and kerasin as optical isomeric substances of the same chemical composition, a view which seemed to receive support by the agreement of their elementary analyses. Further Thierfelder's [1913] substances of the kerasin type also yielded analytical figures which agreed well with the calculated formula and still it was found on hydrolysis that 25 % of the fatty acids consisted of the acid $C_{25}H_{50}O_3$, which is the characteristic component of phrenosin.

It is clear, therefore, that elementary analysis of these highly complex substances is of little value. Up till now the only satisfactory criteria of their purity are furnished by an examination of their optical activity and by the selenite test [Rosenheim, 1914, 1].

Melting point. The behaviour of kerasin on heating and the formation of liquid crystals has been fully described in a previous communication.

Optical activity. Kerasin is laevorotatory in distinction from the dextrorotatory phrenosin and the degree of its laevorotation may serve as an index for the more or less successful removal of phrenosin. Its optical activity increases with rising temperature and varies with the concentration and the nature of the solvent. The most highly purified specimens so far obtained showed an optical activity in pyridine at room temperature of $[\alpha]_D = -3{\cdot}71$ to $-3{\cdot}78°$. In a solution of chloroform containing 10 % pyridine, at 50°, their activity was $[\alpha]_D^{50°} = -4{\cdot}58$ to $-5{\cdot}08°$.

(1) 0·9132 g. in 10 cc. pyridine:
$t = 20°;\ l = 1;\ \alpha = -0{\cdot}25°;\ [\alpha]_D^{20°} = -2{\cdot}74°.$

(2) 0·9004 g. in 10 cc. pyridine:
$t = 18°;\ l = 1;\ \alpha = -0{\cdot}25°;\ [\alpha]_D^{18°} = -2{\cdot}78°.$

(3) 1·0000 g. in 10 cc. pyridine:
$t = 18°;\ l = 1;\ \alpha = -0{\cdot}25°;\ [\alpha]_D^{18°} = -2{\cdot}50°.$

(4) 1·0501 g. in 10 cc. pyridine:
$t = 25°;\ l = 1;\ \alpha = -0{\cdot}39°;\ [\alpha]_D^{25°} = -3{\cdot}71°.$

1·0044 g. in 10 cc. chloroform containing 10 % pyridine:
$t = 50°;\ l = 1;\ \alpha = -0{\cdot}51°;\ [\alpha]_D^{50°} = -5{\cdot}08°.$

Kerasin (Thudichum's method)[1].

1·0052 g. in 10 cc. pyridine:
$t = 25°;\ l = 1;\ \alpha = -0{\cdot}38°;\ [\alpha]_D^{25°} = -3{\cdot}78°.$

0·5025 g. in 10 cc. pyridine-acetone (1 : 1):
$t = 50°;\ l = 1;\ \alpha = -0{\cdot}23°;\ [\alpha]_D^{50°} = -4{\cdot}58°.$

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[1] In connection with some work on the optical activity of brain lipoids Rosenheim and Tebb [1907] examined a sample of kerasin prepared by Thudichum's method and found its optical activity in a 3 % pyridine solution to be $[\alpha]_D^{18°} = -2{\cdot}8°$.

The kerasin fractions examined by Levene and Jacobs [1912, 3] were found by them to be inactive and they therefore proposed for kerasin the name *dl*-cerebrin (phrenosin = *d*-cerebrin). It may be pointed out, however, that they note that "once a sample was obtained from the kerasine fraction that was strongly laevorotatory." Thierfelder's [1913] kerasin fractions were also inactive or even slightly dextrorotatory. It is obvious from the above-mentioned presence of phrenosinic acid in them that they were contaminated with the dextrorotatory phrenosin to the extent of at least 12 %.

Molecular weight. Only one attempt has up till now been made to determine the molecular weight of kerasin by a physical method. Kossel and Freytag [1893] calculate from the rise of boiling point in glacial acetic acid solution the molecular weight of "homocerebrin" (= kerasin) as 986 (945, 1027). These figures can only be taken as approximate since it is well known that the accuracy of Beckmann's method is considerably impaired when applied to substances of high molecular weight which tend to form colloidal solutions. This may also be inferred by the experience of Pearson [1914][1]. Yet it seemed worth while to attempt the estimation of the molecular weight of kerasin by using Barger's [1904] microscopical method based on vapour pressure. Dr A. J. Ewins, who possesses considerable experience with Barger's method, kindly carried out the observations, for which I wish to take this opportunity of thanking him. Employing pyridine as a solvent and benzil as a standard, a solution of 0·080 g. kerasin in 0·500 g. of solvent was found to be between 0·20 and 0·22 mol., *i.e.* molecular weight lies between

$$\frac{0{\cdot}080 \times 1000}{0{\cdot}500 \times 0{\cdot}20} = 800 \text{ and } \frac{0{\cdot}080 \times 1000}{0{\cdot}500 \times 0{\cdot}22} = 727. \quad \text{Mean value} = 763.$$

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[1] A chloroform solution of a mixture of galactosides, prepared by the baryta process, showed an elevation of the boiling point which stands in no relationship to the molecular weight. Pearson draws from his observations the conclusion that the galactosides occur in a chemical combination with sphingomyelin in the so-called "protagon." A single sample of "protagon," of which not even an analysis is given, was examined and failed to show any elevation of the boiling point, whilst a mixture of galactosides and sphingomyelin (prepared from "protagon" by the pyridine method) produced a measurable elevation. Quite apart from the probability that a similar measurable elevation might be found if several samples of "protagon" had been examined, the results recorded are to be expected, if only two components of the complex mixture "protagon" are examined by this method. The author failed to reconstitute the mixture "protagon" in its entirety, since he omitted to add the colloidal substances always present in protagon. These were removed by baryta in the one case and by the pyridine treatment in the other. It is admitted by Pearson that the contentions which were made in favour of the chemical entity of "protagon" were inconclusive and the whole case for "protagon" is now reduced to the value which can be attached to the few incomplete observations quoted. On the other hand the clear and experimentally uncontradicted chemical evidence has established the fact that "protagon" is a heterogeneous mixture.

Considering the size of the molecule, this figure agrees fairly well with the molecular weight ($C_{47}H_{91}O_8N = 797$) as calculated from the cleavage products of kerasin (see above).

It is interesting to note that in the case of phrenosin the method failed owing to the colloidal condition of sufficiently concentrated solutions.

Hydrolysis. This was carried out by the same methods as those employed for phrenosin previously described. Samples of kerasin prepared by the pyridine method as well as by Thudichum's method were hydrolysed and gave identical results. The products of hydrolysis were: (1) lignoceric acid, $C_{24}H_{48}O_2$; (2) sphingosine; and (3) galactose.

(1) *Lignoceric acid.* (*a*) *Methyl ester.* 3·50 g. of kerasin were hydrolysed by boiling under a reflux condenser for 6 hours with 250 cc. methyl alcohol containing 10 % conc. H_2SO_4. On cooling glistening white crystals of the methyl ester of lignoceric acid crystallised out which were filtered and dissolved in a small quantity of methyl alcohol. In order to complete the esterification HCl gas was passed into the hot solution and the ester, which crystallised out on cooling, was filtered, washed and dried *in vacuo*. It weighed 1·27 g. and was recrystallised in two fractions from acetone. The two fractions showed the same melting point of 57°–58°. According to Hell and Hermanns [1880] the methyl ester of lignoceric acid from wood paraffin melts at 56·5°–57° and Kreiling [1888] found the melting point 58° for the methyl ester of lignoceric acid from arachis oil.

The two fractions were combined and again recrystallised from acetone. The melting point remained unaltered. For elementary analysis the substance was dried *in vacuo* at 35°.

(9·075 mg.; 26·1155 mg. CO_2; 10·910 mg. H_2O.)

	Found	Calc. for $C_{23}H_{47}COOCH_3$
C	78·58 %	78·44 %
H	13·44 „	13·18 „

Molecular weight. 0·6044 g. of the ester was saponified by boiling for three hours with alcohol containing 20·42 cc. 0·1N KOH. The excess of potash was then titrated back by means of 0·1N HCl. 16·01 cc. of 0·1N KOH had been used up. Molecular weight = 382. Calc. for $C_{23}H_{47}COOCH_3$: 382.

Another sample of kerasin was hydrolysed in ethyl alcoholic solution. The ethyl ester of lignoceric acid was obtained and melted at 56°, solidifying at 54°. Mixed with lignoceric acid ethyl ester from wood paraffin[1], it showed exactly the same melting and solidifying points.

(*b*) *Free lignoceric acid.* The potassium salt of lignoceric acid was obtained by saponification of the methyl and ethyl esters, filtered and well washed with alcohol and acetone. The fatty acid was set free from the soap by means of dilute hydrochloric acid and freed from mineral acid by repeatedly fusing its watery suspension on the water-bath. It was then extracted with ether, its ethereal solution washed with water, filtered through dry filter paper and evaporated. The white crystalline free acid was dried *in vacuo* and showed a melting point of 81°.

The acid was recrystallised twice from a mixture of light petroleum and acetone and was obtained in silky white crystals, forming a loose powder which assumed a wax-like consistency when compressed in an agate mortar.

Melting point. The substance had a sharp melting point of 81·0° and solidified at 74·5°–73·5°. The melting point of lignoceric acid is given in the literature as 80·5°–81° [Hell and Hermanns, 1880; Kreiling, 1888]. Samples were mixed with Hell's lignoceric acid and with lignoceric acid prepared by myself from arachis oil according to Kreiling. The melting points of the various mixtures were identical at 81·0°.

Optical activity. A 10 % solution of the acid in chloroform at 25° was found to be inactive.

Elementary analysis.

(1) 0·0745 g.; 0·2136 g. CO_2; 0·0862 g. H_2O.
(2) 5·145 mg.; 14·810 mg. CO_2; 5·848 mg. H_2O.

	(1)	(2)	Calc. for $C_{24}H_{48}O_2$
C	78·19 %	78·50 %	78·17 %
H	12·95 „	12·72 „	13·13 „

Molecular weight. (*a*) 0·2000 g. of the acid was titrated in light petroleum solution with 0·1N alcoholic potash (phenolphthalein as indicator) and required 5·39 cc. 0·1N KOH.

(*b*) 0·2205 g. of a different preparation (Thudichum's) required 5·97 cc. 0·1N KOH.

	(*a*)	(*b*)	Calc. for $C_{24}H_{48}O_2$
Molecular weight	371	369	368

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[1] Through the kindness of Prof. Hell, the discoverer of lignoceric acid [1880], I came into the possession of samples of his original lignoceric acid and its ethyl ester, which also served for the identification of lignoceric acid from kidney lipoids [Rosenheim and MacLean, 1915].

The acid recovered from the potassium salts after titration showed the same melting point as before.

Silver salt. The acid was dissolved in alcohol and the silver salt precipitated by means of an alcoholic solution of silver nitrate. A drop of dilute alcoholic ammonia was added and the silver salt, which is not very sensitive to light, was filtered, washed with boiling alcohol and dried in a dark desiccator.

0·1208 g. gave 0·0273 g. Ag = 22·59 %,
Calc. for $C_{23}H_{47}COOAg$: 22·74 %.

Lead salt. The acid was dissolved in methyl alcohol and precipitated with a solution of lead acetate in methyl alcohol. A small amount of dilute methyl alcoholic ammonia was added to complete the precipitation. The lead salt was filtered, washed with hot acetone and dried *in vacuo*. It formed a white heavy powder which melted at 117°. The melting point of lead lignocerate is given as 117° by Hell and Hermanns [1880]. Another preparation was made from lignoceric acid obtained by the hydrolysis of Thudichum's kerasin.

(1) 0·1804 g. gave 0·0577 g. lead sulphate.
(2) 0·1508 g. gave 0·0482 g. lead sulphate.

	Pb	Calc. for $(C_{24}H_{47}O_2)_2Pb$
(1)	21·84 %	
(2)	21·83 ,,	21·99 %

(2) *Sphingosine.* After the removal of lignoceric acid the alcoholic hydrolysate was diluted with water, kept on the water-bath for some time and the alcohol finally evaporated. Just as in the case of phrenosin, it was found that besides sphingosine a certain amount of methyl derivatives of sphingosine had been formed during the methyl alcoholysis.

(*a*) *Sphingosine sulphate* was separated from this mixture by its relative insolubility in cold alcohol, in which methylsphingosine sulphate is soluble. After repeated purification by conversion into the free base, sphingosine sulphate was obtained as a white hygroscopic powder, showing a melting point of about 245°, and decomposing with effervescence at 250°. For further identification the amino-nitrogen was estimated by van Slyke's method, using the micro-apparatus recently described.

Amino-nitrogen estimation. A solution of sphingosine sulphate in glacial acetic acid was employed, containing 0·1484 g. of the substance in 10 cc. solution. 2 cc. were used for each experiment and the nitrogen evolution was found to be complete in 25–30 minutes. After making the necessary correction, 2·01 cc. nitrogen at 763 mm. and 15° C. were obtained from 2 cc. of the solution.

	Found	Calc. for $(C_{17}H_{33}O_2NH_2)_2H_2SO_4$
N	3·96 %	4·19 %

(*b*) The *easily soluble sulphate* was converted into the free base by the addition of potash to its alcoholic solution. After addition of water the base was extracted with ether and it crystallised at once on evaporation of the solvent. The free base was dissolved in absolute ether and gaseous hydrochloric acid was passed into the solution. It solidified to a mass of crystals of the hydrochloride which was filtered off and washed with ether and acetone. The substance was recrystallised from a mixture of alcohol and acetone; it crystallised in transparent plates, resembling cholesterol crystals.

(0·0700 g.; 0·1644 g. CO_2; 0·0707 g. H_2O.)

	Found	Calc. for monomethylsphingosine hydrochloride	Calc. for dimethylsphingosine hydrochloride
C	64·05 %	64·32 %	65·18 %
H	11·30 „	11·40 „	11·52 „

The substance melted sharply at 141°, whilst dimethylsphingosine hydrochloride melts at 132°. The low carbon percentage and the higher melting point suggest that the substance represents a monomethylsphingosine hydrochloride.

In this connection it may be mentioned that Thierfelder [1913] also obtained on hydrolysis of various kerasin fractions a hydrochloride which showed the abnormal melting point of 139°. As the material employed for hydrolysis still admittedly contained phrenosin, it is possible that the substance, which was considered as dimethylsphingosine, consisted of a mixture of this substance with monomethylsphingosine. This question, which is of importance for the elaboration of the constitutional formula of kerasin, requires further investigation.

(3) *d-Galactose*. The carbohydrate contained in kerasin has up till now not been definitely identified. Thudichum assumed it to be identical with cerebrose (galactose) which he had obtained from phrenosin. I obtained the carbohydrate in the crystallised condition and identified it with galactose by oxidation to mucic acid and by its characteristic methylphenylhydrazone.

The hydrolysate of 3·50 g. kerasin, from which the fatty acid and bases had been removed as above described, was made up to 250 cc. and examined polarimetrically. Actual rotation measured in 2 dm. tube = + 0·48°. Whence galactose = 21·19 %. Calc. for 1 mol. galactose from $C_{47}H_{91}O_8N$ = 22·6 %. Considering the unavoidable losses in the lengthy operations of the estimation, the value found agrees well with the calculated figure.

From one-half of the solution the carbohydrate was obtained by removing the sulphuric acid by means of barium carbonate and concentrating the filtrate *in vacuo*.

The residue was taken up in methyl-alcohol and the sugar recrystallised from water, from which it separated in the forms characteristic of galactose. A small quantity of the crystals was oxidised with dilute nitric acid according to Tollens. The mucic acid obtained showed the melting point of 190° and was converted into the ammonium salt by means of ammonium carbonate. The dry ammonium salt gave on heating a strong pyrrole reaction.

To the other half of the final hydrolysate solid sodium acetate was added until the reaction ceased to be acid towards Congo-paper. A few drops of methylphenylhydrazine were then added and the hydrazone separated and recrystallised as described above under phrenosin. The hydrazone showed a sharp melting point of 191°.

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SUMMARY.

1. The galactoside phrenosin prepared from brain by the pyridine method is identical with Thudichum's phrenosin.

2. The substances described by various authors as pseudo-cerebrin cerebrin, cerebron and *d*-cerebrin are identical with phrenosin.

3. Phrenosin is dextrorotatory and furnishes on hydrolysis: (1) phrenosinic acid ($C_{25}H_{50}O_3$), (2) sphingosine, and (3) galactose.

4. Kerasin is laevorotatory and furnishes on hydrolysis: (1) lignoceric acid ($C_{24}H_{48}O_2$), (2) sphingosine, and (3) galactose.

5. Constitutional formulae for the two galactosides of brain are suggested.

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XVI. RECHERCHES SUR L'ANAPHYLAXIE PAR LA DIGLYCYLGLYCINE.

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INTRODUCTION.

Au cours de ses recherches sur la séroanaphylaxie du lapin, Arthus [1909, 1910] a soumis des lapins à 5 injections de 5 cm.^{3} chacune de solution aqueuse de glycocolle à 5 %. Quinze jours après la dernière injection, Arthus a introduit dans la veine auriculaire 4 cm.^{3} soit de sérum de cheval, soit de solution aqueuse de glycocolle à 5 %. Dans le premier cas, l'on obtient les réactions caractéristiques du choc anaphylactique, dans le second pas. Ces expériences mettaient en évidence deux faits très intéressants: 1° on peut anaphylactiser des lapins par des dérivés très éloignés des protéines; 2° une substance peut être anaphylactisante sans être toxique.

D'autre part, l'un de nous a montré [Zunz, 1911] que certaines protéoses (hétéroalbumose, protoalbumose, synalbumose) sensibilisent le cobaye et le lapin. Seules, l'hétéroalbumose et la protoalbumose déchaînent le choc anaphylactique chez les animaux préparés par l'une des trois protéoses précédentes. Chez le cobaye, le sérum de bœuf déchaîne encore plus aisément le choc que la protoalbumose ou l'hétéroalbumose, tandis que le sérum de cheval ne possède pas cette propriété.

Bien d'autres chercheurs se sont demandés, en ces dernières années, si l'on parvient à anaphylactiser les animaux au moyen de dérivés des protéines. Abderhalden a publié des résultats positifs [1914] obtenus chez des cobayes soumis à l'injection de polypeptides déjà assez compliqués. Mais Friedberger [1910; Friedberger et Joachimoglu, 1914] conteste les résultats d'Abderhalden et ceux de l'un de nous (Z.). Il estime qu'on n'est pas parvenu jusqu'à présent à anaphylactiser des animaux par des protéoses, des peptides et, par conséquent, encore moins par des acides aminés.

Il serait trop long de passer en revue tous les travaux consacrés à cette question. Quoi qu'il en soit, en présence des résultats positifs obtenus par Arthus avec le glycocolle, on pouvait se demander si, en employant de la diglycylglycine ($NH_2.CH_2.CO.NH.CH_2.CO.NH.CH_2.COOH$), l'on n'arriverait pas à déchaîner au moyen de ce produit le choc anaphylactique chez des lapins préparés par des injections sous-cutanées de cette substance.

A cet effet, nous avons préparé, lors de chaque série d'injections, une solution fraîche de diglycylglycine à 2 % dans de la solution physiologique à 1 %. On a effectué une fois par semaine les injections préparantes sous la peau. Après trois à six injections, on a injecté dans la veine auriculaire du lapin la diglycylglycine ou le sérum frais de cheval huit jours après la dernière injection préparante.

On a enregistré la pression artérielle à l'aide d'un hémodynamographe de Verdin communiquant avec la carotide, la respiration à l'aide d'un tambour enregistreur de Marey, commandé par un pneumographe à double tambour appliqué sur la région diaphragmatique.

Pour apprécier la coagulabilité, nous avons rejeté les premiers $cm.^3$ de sang carotidien souillés par mélange avec le liquide antihémostatique. Nous avons ensuite recueilli directement le sang de la carotide dans une capsule en porcelaine, sans lui permettre de couler sur la plaie. Nous avons noté le temps nécessaire, 1° à l'apparition des premiers flocons fibrineux, 2° à la coagulation en bloc.

EXPÉRIENCES.

I. *Effets de l'injection intraveineuse de diglycylglycine chez des lapins neufs.*

Il était nécessaire de reconnaître au préalable les effets éventuels de l'injection intraveineuse de diglycylglycine chez des lapins neufs. Nous résumons trois expériences de ce genre.

Expérience I. Un lapin, de 2300 gr., reçoit, en injection intraveineuse, 2·5 $cm.^3$ de solution à 2 % de diglycylglycine, soit 5 centigr. de ce peptide. Ceci correspond à 2·15 centigr. de diglycylglycine par kilogramme.

La pression carotidienne s'élève d'abord légèrement; elle passe de 10·2 centimètres de mercure à 10·6, ½ minute après l'injection. Puis survient une chute graduelle et la pression atteint son minimum (8·4 centimètres) 14 minutes après l'injection pour ne plus remonter pendant les 10 minutes suivantes. Il s'est donc produit une chute de 1·8 centimètre de mercure.

La fréquence respiratoire, qui oscillait entre 42 et 55 mouvements par minute, a subi une légère accélération (60 à 74 mouvements par minute) de la dixième à la douzième minute consécutive à la fin de l'injection. A cette période, les mouvements respiratoires ont été peu amples. Ils ont ensuite repris le rythme initial.

Il ne s'est pas produit d'expulsion de bols fécaux.

Le sang carotidien, prélevé 25 minutes après la fin de l'injection, a montré les premiers flocons au bout de 20 minutes. La coagulation a été achevée 5 minutes plus tard.

Expérience II. Un lapin, de 1865 gr., reçoit, en injection intraveineuse, 10 centigr. de diglycylglycine, soit 5·3 centigr. par kilogramme.

A part une élévation passagère de 0·6 centimètre de mercure pendant l'injection, la pression artérielle s'est maintenue entre 9·8 et 10 centimètres de mercure.

Le rythme respiratoire rapide avant l'injection (82 mouvements par minute) subit un ralentissement graduel et se maintient entre 35 et 40 mouvements par minute, à partir de la quatorzième minute consécutive à l'injection.

Il y a eu expulsion de nombreux bols fécaux.

Le sang carotidien, prélevé 23 minutes après la fin de l'injection, a montré les premiers flocons au bout de 12 minutes. La coagulation complète a exigé 28 minutes.

Expérience III. Un lapin, de 1885 gr., reçoit, en injection intraveineuse, 10 centigr. de diglycylglycine, soit 5·31 centigr. par kilogramme.

L'élévation passagère lors de l'injection est plus accentuée ici que dans les expériences I et II. La pression carotidienne passe de 10 centimètres de mercure à 12·8, ½ minute après l'injection. Puis survient une chute rapide de la pression revenue à son niveau initial 1½ minute après l'injection. La pression s'abaisse ensuite lentement et atteint son niveau le plus bas (9·2 centimètres de mercure) 21 minutes après l'injection. L'abaissement par rapport au niveau initial correspond alors à 0·8 centimètre.

Comme dans l'expérience II, le rythme respiratoire, fort rapide au moment de l'injection (94 par minute), subit un ralentissement graduel et ne représente plus que 35 mouvements par minute à la fin de l'expérience.

Il y a eu expulsion de 30 bols fécaux.

Le sang carotidien, prélevé 24 minutes après la fin de l'injection, a montré les premiers flocons au bout de 30 minutes. La coagulation complète a exigé 50 minutes.

Des trois expériences précédentes, il résulte que, par elle-même, l'injection intraveineuse de diglycylglycine a amené, deux fois sur trois, une chute peu accentuée et lente de la pression artérielle, n'atteignant pas 2 cm. de mercure. Le rythme respiratoire ne subit pas de modifications ou tend à se ralentir. La coagulation subit un retard. Il y a lieu de rapprocher ce dernier résultat des faits suivants établis par Zunz et György [1914] : Des doses très faibles de diglycylglycine accélèrent la coagulation du plasma oxalaté (de chien ou de lapin) recalcifié et parviennent même à faire coaguler du plasma insuffisamment recalcifié. Mais un excès de ce polypeptide entrave au contraire la coagulation du plasma oxalaté recalcifié. On ne doit donc pas interpréter le retard de la coagulation, à la suite de l'injection intraveineuse de diglycylglycine, comme un symptôme de choc anaphylactique.

II. *Effets de l'injection intraveineuse de diglycylglycine chez des lapins ayant subi au préalable des injections sous-cutanées de ce peptide.*

Passons maintenant aux expériences proprement dites. Recherchons tout d'abord les effets de l'injection intraveineuse de diglycylglycine chez des lapins préparés au moyen d'injections sous-cutanées de ce peptide, répétées à sept jours d'intervalle. A titre d'exemple, nous résumons ci-dessous trois expériences ainsi effectuées.

Expérience IV. Un lapin, de 1825 gr., reçoit 3 injections sous-cutanées de diglycylglycine. On lui a injecté en tout 9 centigrammes de ce peptide en 22 jours. Sept jours après la dernière injection sous-cutanée, on introduit, dans la veine de l'oreille, en ½ minute, 5 cm.³ de solution à 2 % de diglycylglycine, soit 10 centigr. de ce peptide. Ceci correspond à 5·48 centigr. par kilogramme.

La pression artérielle s'abaisse graduellement de 10·8 centimètres de mercure avant l'injection à 8·2 quinze minutes après l'injection. La chute de pression atteint donc 2·6 centimètres à ce moment, où l'on a malheureusement interrompu l'expérience en injectant du sérum (voir plus loin, expérience XII).

On constate, en outre, une accélération passagère des mouvements respiratoires deux minutes après la fin de l'injection (80 par minute au lieu de 40 à 50 auparavant et par la suite). Ce sont là les signes d'une réaction anaphylactique nette, quoique peu intense.

Par contre, il n'y a pas eu d'expulsion de matières fécales.

Expérience V. Un lapin, de 2300 gr., reçoit 5 injections sous-cutanées de diglycylglycine. On lui a injecté en tout 15 centigrammes de ce peptide en 36 jours. Sept jours après la dernière injection sous-cutanée, on introduit, dans la veine de l'oreille, en ½ minute, 4 cm.³ de solution à 2 % de diglycylglycine, soit 8 centigrammes de ce peptide. Ceci correspond à 3·48 centigrammes par kilogramme.

La pression artérielle s'abaisse graduellement de 11·0 centimètres de mercure à 9·5. Cette chute maxima de 1·5 cm. est atteinte au bout de 13 minutes. La pression remonte lentement à partir de la seizième minute. L'abaissement de pression ressemble à celui observé dans l'expérience I chez un lapin non soumis à des injections préparantes sous-cutanées de diglycylglycine. En outre, le chiffre de mouvements respiratoires s'est maintenu pendant tout le cours de l'expérience entre 70 et 90 par minute. Par contre, il y a eu émission de bols fécaux.

Expérience VI. Un lapin, de 1915 gr., reçoit 6 injections sous-cutanées de diglycylglycine. On lui a injecté en tout 18 centigrammes de ce peptide en 43 jours. Sept jours après la dernière injection sous-cutanée, on a introduit, dans la veine de l'oreille, en ½ minute, 5 cm.³ de solution à 2 % de diglycylglycine, soit 10 centigrammes de ce peptide. Ceci correspond à 5·22 centigrammes par kilogramme.

Il se produit une chute de pression atteignant 4·5 centimètres de mercure au bout de 16 minutes; la pression passe de 12·4 à 7·9 centimètres; elle remonte ensuite lentement. La respiration n'a pas subi de modifications appréciables. Aussitôt après l'injection, on a constaté, à travers la paroi abdominale, des mouvements péristaltiques très intenses des intestins, qui ont entraîné, au bout d'un quart d'heure, l'expulsion de 28 bols fécaux. Ce phénomène et l'intensité de la chute de pression permettent de conclure à une réaction anaphylactique nette.

Du sang carotidien, prélevé 20 minutes après la fin de l'injection, a montré les premiers flocons au bout de 25 minutes. La coagulation complète a exigé 30 minutes.

Les expériences IV et VI démontrent qu'il est possible d'anaphylactiser le lapin par des injections sous-cutanées de diglycylglycine. Trois injections pratiquées à sept jours d'intervalle suffisent déjà à cet effet, mais un plus grand nombre d'injections paraît préférable.

La diglycylglycine parvient à provoquer les symptômes du choc anaphylactique chez les lapins ainsi préparés.

Si l'on n'a pas obtenu de réaction anaphylactique dans l'expérience V, cela tient peut-être non pas à une quantité insuffisante d'injections préparantes, mais plutôt à la quantité trop faible de diglycylglycine introduite dans le sang.

Dans les expériences effectuées par Arthus avec le glycocolle, chaque lapin a été soumis à 5 injections de 5 cm.³ d'une solution aqueuse de glycocolle à 5 %, pratiquées sous la peau à sept jours d'intervalle. Il a donc reçu 125 centigrammes de glycocolle en 36 jours. Même en tenant compte de la différence entre les poids moléculaires de la diglycylglycine et du glycocolle et du plus grand degré de complexité moléculaire du premier de ces composés cristallins, on doit se demander, s'il n'y aurait pas eu intérêt, pour mieux mettre en évidence les phénomènes du choc anaphylactique, à utiliser de plus fortes doses de diglycylglycine, tant lors des injections sous-cutanées préparantes que lors de l'injection intraveineuse d'essai.

D'autre part, Arthus n'a jamais réussi à obtenir la moindre modification respiratoire ou circulatoire en injectant 25 centigrammes de glycocolle dans la veine auriculaire d'un lapin soumis à 5 injections préparantes de cet acide aminé. L'injection intraveineuse subséquente de sérum chez un tel animal démontre qu'il était néanmoins parfaitement anaphylactisé. Or, à la dose de 10 centigrammes, la diglycylglycine parvient déjà très bien à provoquer les phénomènes du choc anaphylactique. Nous nous trouvons donc en présence d'une différence essentielle entre le glycocolle et le peptide formé par l'union de trois molécules de cet acide aminé. Le glycocolle n'agit que comme sensibilisateur, alors que la diglycylglycine est à la fois sensibilisatrice et déchaînante.

III. *Effets de l'injection intraveineuse de sérum chez des lapins ayant subi au préalable des injections sous-cutanées de diglycylglycine.*

Résumons les résultats de trois expériences relatives à ce point.

Expérience VII. Un lapin, de 1930 gr., reçoit 5 injections sous-cutanées de diglycylglycine. On lui a injecté en tout 15 centigrammes de ce peptide en 36 jours. Sept jours après la dernière injection sous-cutanée, on introduit, dans la veine de l'oreille, en ½ minute, 10 centimètres cubes de sérum frais de cheval.

La pression artérielle s'abaisse, en 10 minutes, de 9·3 centimètres de mercure à 8 centimètres; après cette chute de 1·3 centimètre, la pression remonte rapidement; elle est déjà bien près du niveau initial 12 minutes après l'injection.

La respiration, fort rapide avant l'injection (110 mouvements par minute), se ralentit quelque peu après celle-ci (80 à 90 mouvements par minute).

Il y a en expulsion de 6 bols fécaux après l'injection.

Expérience VIII. Un lapin, de 2550 gr., reçoit 6 injections sous-cutanées de diglycylglycine. On lui a ainsi injecté en tout 18 centigrammes de peptide en 43 jours. Sept jours après la dernière injection sous-cutanée, on introduit, dans la veine de l'oreille, en ½ minute, 5 cm.³ de sérum frais de cheval.

En 4 minutes, la pression tombe de 11 centimètres de mercure à 6·3 centimètres. Après cette chute de 4·7 centimètres, la pression remonte jusqu'à 9·4 centimètres, à la quatorzième minute consécutive à l'injection, pour redescendre de nouveau quelque peu ensuite et ne plus atteindre que 8 centimètres à la seizième minute et 8·1 à la dix-septième.

Avant l'injection, on compte 41 mouvements respiratoires par minute. Le nombre des mouvements s'élève fort vite et atteint 128 la deuxième minute après l'injection, 125 la troisième; la respiration est donc devenue trois fois plus rapide qu'avant l'injection; à ce moment, il y a de la dyspnée manifeste. Puis le rythme respiratoire revient vite à un chiffre oscillant entre 46 et 64 par minute.

Il n'y a pas eu d'émission de matières fécales.

Expérience IX. Un lapin, de 1980 gr., reçoit 6 injections sous-cutanées de diglycylglycine. On lui a injecté en tout 18 centigrammes de ce peptide en 43 jours. Sept jours après la dernière injection sous-cutanée, on introduit, dans la veine de l'oreille, en ½ minute, 5 centimètres cubes de sérum frais de cheval.

Cinq minutes après la fin de l'injection, la pression artérielle est tombée de 10·8 à 7·6 centimètres de mercure, c'est-à-dire a subi une chute de 3·2 centimètres. La chute maxima n'est néanmoins atteinte qu'au bout de 18 minutes; elle correspond à 4·2 centimètres de mercure.

Bien que le rythme respiratoire fût déjà très rapide (97 mouvements par minute) avant l'injection de sérum, celle-ci a cependant provoqué un accroissement notable de la fréquence des mouvements respiratoires. Cette accélération respiratoire a atteint son maximum (170 mouvements par minute) les quatrième et cinquième minutes après l'injection. La fréquence respiratoire a ensuite diminué; elle correspondait à 125 mouvements par minute de la huitième à la dixième minute après l'injection, de 80 à 90 jusqu'à la quinzième minute, puis est descendue à 52 mouvements à la dix-huitième minute pour remonter à 70 à la dix-neuvième minute.

Il n'y a pas eu d'expulsion de matières fécales.

Du sang carotidien prélevé 20 minutes après la fin de l'injection, a montré les premiers flocons au bout de 16 minutes. La coagulation a été achevée 24 minutes après le prélèvement.

Les expériences VIII et IX faites chez des lapins soumis à 6 injections de diglycylglycine permettent de reconnaître que le sérum frais de cheval, introduit dans le torrent circulatoire de ces animaux, amène chez eux les symptômes du choc anaphylactique, tout comme chez les lapins préparés par des injections hypodermiques de glycocolle. Si la réaction est moins intense dans les expériences VIII et IX que dans les recherches effectuées par Arthus chez les animaux soumis au préalable à des injections de glycocolle, cela tient peut-être aux quantités relativement faibles de peptide reçues par nos lapins.

Le résultat négatif de l'expérience VII, effectuée chez un animal soumis seulement à 5 injections de diglycylglycine, tient peut-être à cette cause. Peut-être aussi la dose de sérum employée lors de l'injection intraveineuse d'essai (10 cm.^{3} au lieu de 5 dans les expériences VIII et IX) était-elle trop considérable. Enfin, il ne faut pas perdre de vue que, dans toutes les recherches relatives à l'anaphylaxie, on signale des animaux qui, pour un motif ou un autre, souvent ignoré de l'investigateur, ne réagissent pas comme la généralité des sujets en expérience.

Quoi qu'il en soit, les expériences IV à IX nous amènent à conclure qu'on parvient à anaphylactiser le lapin par un nombre suffisant d'injections sous-cutanées de diglycylglycine. On met aisément en évidence chez ces animaux la réaction anaphylactique par l'injection intraveineuse, soit de diglycylglycine, soit de sérum sanguin.

IV. *Effets de l'injection intraveineuse de diglycylglycine consécutive à l'injection intraveineuse de sérum chez des lapins ayant subi au préalable des injections sous-cutanées de diglycylglycine.*

A titre complémentaire, nous croyons utile de relater les deux essais suivants, dans lesquels des lapins, préparés par une série d'injections sous-cutanées de diglycylglycine, ont reçu, quelque temps après l'introduction de sérum frais de cheval dans le sang, une injection intraveineuse de diglycylglycine.

Expérience X. Dix-sept minutes après l'injection intraveineuse de sérum chez le lapin utilisé lors de l'expérience VII, lapin soumis au préalable à 5 injections sous-cutanées de diglycylglycine, on introduit, dans la veine de l'oreille, 5 cm.^{3} de solution à 2 % de diglycylglycine, soit 10 centigrammes de ce peptide. Ceci correspond à 5·19 centigrammes par kilogramme.

L'injection intraveineuse de diglycylglycine a amené, en 9 minutes, la pression artérielle de 9·3 à 7·5 centimètres de mercure. Cet abaissement, de 1·8 centimètre, est plus considérable que celui obtenu chez ce même lapin sous l'influence de l'injection intraveineuse de sérum (1·3 centimètre de mercure au bout de 10 minutes), mais il ne dépasse néanmoins pas celui obtenu dans l'expérience I sous l'influence d'une injection intraveineuse de diglycylglycine chez un lapin non soumis au préalable à des injections sous-cutanées de ce peptide. La pression artérielle remonte ensuite de 0·2 centimètre de mercure.

La respiration (110 mouvements par minute) s'est ralentie après l'injection de diglycylglycine; elle a montré le minimum de fréquence, du reste encore assez élevé (65 mouvements par minute), 2 à 5 minutes après la fin de cette injection, puis elle a subi peu à peu une accélération qui l'a amenée, de la neuvième à la onzième minute consécutives à l'injection de diglycylglycine à un chiffre de 100 à 115 mouvements par minute pour redescendre ensuite au chiffre de 90 à 95 mouvements par minute.

Immédiatement après l'injection de diglycylglycine, il y a eu émission de 12 bols fécaux.

Du sang carotidien, prélevé 15 minutes après la fin de cette injection, a montré les premiers flocons au bout de 12 minutes. La coagulation a été achevée 2 minutes plus tard, soit 14 minutes après le prélèvement.

Expérience XI. Dix-huit minutes après l'injection intraveineuse de sérum chez le lapin utilisé lors de l'expérience VIII, lapin soumis au préalable à 6 injections sous-cutanées de diglycylglycine, on introduit, dans la veine de l'oreille, 5 cm.^{3} de solution à 2 % de diglycylglycine, soit 10 centigrammes de ce peptide. Ceci correspond à 3·92 centigrammes par kilogramme.

La pression artérielle a passé ½ minute après l'injection de diglycylglycine de 8·1 à 8·8 centimètres de mercure, puis est revenue à 8·1 centimètre. Il n'y a donc pas eu d'effet sur la pression artérielle.

Par contre, le nombre des mouvements respiratoires a passé de 52 par minute à 100, chiffre atteint 2 minutes après l'injection, puis est revenu à 60 la cinquième minute consécutive à l'injection.

Il n'y a pas eu d'émission de matières fécales.

Les expériences X et XI semblent montrer qu'une injection intraveineuse de diglycylglycine, consécutive à une injection intraveineuse de sérum, a les mêmes effets que celle-ci. On observe, dans les deux cas, soit une réaction anaphylactique (expériences VIII et XI), soit seulement une chute de la pression artérielle d'intensité insuffisante pour être considérée comme un symptôme du choc anaphylactique (expériences VII et X).

V. *Effets de l'injection intraveineuse de sérum consécutive à l'injection intraveineuse de diglycylglycine chez des lapins soumis au préalable à des injections sous-cutanées de ce peptide.*

Expérience XII. Quinze minutes après l'injection intraveineuse de diglycylglycine chez le lapin qui a servi à l'expérience IV, lapin soumis à 3 injections sous-cutanées préalables de ce peptide, on introduit, dans la veine de l'oreille, 5 cm.^{3} de sérum frais de cheval. L'injection intraveineuse de diglycylglycine avait amené les symptômes d'une réaction anaphylactique nette, mais peu intense.

L'injection de sérum fait monter la pression de 8·2 à 8·6 centimètres de mercure, puis l'abaisse, au bout de trois minutes, à 7·9 centimètres, niveau auquel elle reste ensuite.

Le nombre de mouvements respiratoires par minute s'abaisse de 54 à 44.

Il n'y a pas eu d'expulsion de matières fécales.

Du sang carotidien, prélevé 6 minutes après l'injection de sérum, a montré les premiers flocons au bout de 10 minutes. La coagulation a été achevée 5 minutes plus tard, soit 15 minutes après le prélèvement.

Expérience XIII. Dix-huit minutes après l'injection intraveineuse de diglycylglycine chez le lapin qui a servi à l'expérience V, lapin soumis à 5 injections sous-cutanées préalables de ce peptide, on introduit, dans la veine de l'oreille, 3 cm.^{3} de sérum frais de cheval. Quatre minutes plus tard, on injecte, dans cette même veine, 5 cm.^{3} de ce sérum. L'injection intraveineuse de diglycylglycine a amené, chez cet animal, une légère baisse de pression, sans accélération respiratoire concomitante.

La première injection de sérum a amené une légère élévation de la pression artérielle qui a passé de 9·9 à 10·5 centimètres de mercure. Le nombre de mouvements respiratoires s'est maintenu, comme avant l'injection, de 80 à 90 par minute. Il n'y a pas eu d'expulsion de matières fécales.

La seconde injection de sérum a fait monter la pression artérielle de 10·5 à 10·8 centimètres de mercure mais la pression est revenue de suite à 10·5 centimètres. La respiration a subi une accélération à partir de la troisième minute consécutive à l'injection ; elle a atteint son maximum de fréquence (120 mouvements par minute) les quatrième et cinquième minutes consécutives à l'injection ; l'accélération a persisté jusqu'à la dixième minute après l'injection.

Immédiatement après cette seconde injection, il y a eu expulsion de 69 bols fécaux.

Du sang carotidien, prélevé 12 minutes après la seconde injection de sérum, a montré les premiers flocons au bout de 20 minutes. La coagulation a été achevée 5 minutes plus tard, soit 25 minutes après le prélèvement.

L'injection intraveineuse de sérum effectuée quelque temps après l'injection intraveineuse de diglycylglycine a amené la réaction anaphylactique lorsque l'injection première de peptide ne l'avait pas produite (expérience XIII) et ne l'a pas entraînée au contraire lorsque l'introduction de diglycylglycine dans le sang l'avait déjà provoquée (expérience XII). Il se pourrait certes qu'on se trouve là en présence d'un simple hasard expérimental. On est néanmoins tenté d'admettre que le sérum n'agit que lorsque les effets de l'injection intraveineuse de diglycylglycine ont été nuls ou peu marqués.

Résumé.

On parvient à anaphylactiser le lapin par des injections sous-cutanées de diglycylglycine, répétées à sept jours d'intervalle. Trois injections suffisent déjà parfois à cet effet, mais un plus grand nombre est préférable pour obtenir des résultats très nets.

L'injection intraveineuse, soit de diglycylglycine, soit de sérum frais de cheval, amène, chez les lapins ainsi préparés, les symptômes du choc anaphylactique: chute de la pression artérielle, accélération de la respiration, exagération des mouvements de l'intestin. Les divers symptômes ne coexistent pas dans tous les cas.

La chute de la pression artérielle doit dépasser deux centimètres de mercure pour représenter une réaction anaphylactique, car l'injection intraveineuse de diglycylglycine chez un lapin n'ayant pas été soumis à des injections préalables sous-cutanées de ce peptide amène parfois une chute peu accentuée et très lente de cette pression.

L'injection intraveineuse de diglycylglycine chez un lapin neuf ou bien n'entraîne aucun effet sur le rythme respiratoire ou bien tend à le ralentir.

La diglycylglycine ayant par elle-même une action sur la coagulation du sang, l'étude de la coagulabilité sanguine ne peut pas servir de critérium d'une réaction anaphylactique chez les animaux préparés par les injections sous-cutanées de ce peptide.

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XVII. ADSORPTIVE STRATIFICATION IN GELS.

By SAMUEL CLEMENT BRADFORD.

From the Science Museum.

(*Received March 15th, 1916.*)

In a previous paper [Bradford, 1916] the author proposed the theory that the Liesegang phenomenon might be due to gradual adsorption of the solute in the gel by the growing precipitate, so that, the adjacent layer of gel presently becoming exhausted of solute, the reacting substance would be able to diffuse further into the gel before commencing to form a fresh stratum of precipitate. Hatschek [1914] had proved that Ostwald's hypothesis of metastable solutions was not of general application. Moreover experiments with gels containing additional quantities of the soluble products of the double decomposition, which had been assumed by some to play an essential part in the formation of the strata, showed no appreciable effect on their character or separation. The theory affords an explanation of the fact observed by Pringsheim that diffusion takes place from the hypertonic into the hypotonic region, whether liquid or gel, and that, as recorded by the author, in the absence of a precipitate, mutual interdiffusion results. By working with a coloured solute, such as potassium chromate, in the gel, the gradual exhaustion of the zone near the precipitate could be observed. This is shown in Fig. 1 (Plate I) which represents a tube prepared with N/20 K_2CrO_4 in 1 % agar on which a N/5 solution of $Pb(NO_3)_2$ was poured. Stratification became more evident lower down the tube, but the yellow colour was then less marked. Experiments in 1 % agar in which columns of fuller's earth were embedded above dye solutions showed similar adsorption, though not so intense, since the surface was not being constantly renewed by precipitation.

The agar gel is easily prepared by boiling 10 g. of agar sticks in a litre of water and straining through muslin while hot into a wide-mouthed bottle. The resulting gel should be perfectly clear, otherwise stratification may be hindered, or altogether prevented. When required for use a quantity of the jelly is warmed in a beaker on the water-bath and strained into stoppered test-tubes graduated to contain 15 and 25 cc. The tubes are previously prepared by adding the requisite quantity of standard solutions of the reagents intended, so that, when filled to the 15 cc. mark with agar, the gel will be of the desired concentration. When the gel is set, standard solutions are poured above up to the 25 cc. mark. The concentrations which frequently give the best results are about N/20 for the gel and N to N/5 for the solution above.

Experiments have been made with a variety of insoluble substances. Potassium iodide treated with lead nitrate, and manganese sulphate with liver of sulphur, are noticeable as rapidly producing clearly defined strata. The structure of the manganese sulphide in Fig. 2 (Plate I) closely resembles that of certain limestone formations from Fulwell Hill district. Precipitates which refuse to stratify when prepared in straight tubes, generally yield the desired result when the reacting solutions are poured into the two limbs of a U-tube half filled with agar gel. Copper carbonate and ferrocyanide, cobalt ferricyanide and barium sulphate are examples. As the concentration of the gel falls slowly in U-tube experiments, owing to the reserve of the more dilute reagent in solution above the gel, the discs of precipitate are formed at nearly equal intervals after the first two or three. A necessary condition for the production of clearly defined strata appears to be that the reacting solutions should not be too strong. From his experiments on the adsorption of soluble substances by filter paper Evans [1906] showed that the proportion of solute removed increases with dilution, but that, in the cases examined, no adsorption could be observed in strong solution. After diffusing through a column of gel in a U-tube, the concentrations of the meeting solutions are reduced to the necessary dilution, while sufficient quantities of the reagents are available to produce well defined discs. So far as has been observed, under these conditions stratification is well developed.

The distances between the layers should depend mainly upon C, the concentration of the gel, and the ratio U/C, U being the concentration at the boundary surface between the solid and gel phases at the temperature of the experiment. The distances should therefore be characteristic of the solute in the gel. They should also be affected by the permeability of the precipitate to the reagent diffusing through, and, to some extent, by the rate of diffusion of the reagent in the gel. If the adsorption of one solute is affected by the presence of a considerable quantity of another, as seems to be the case, stratification would also be influenced by increasing the concentration of the

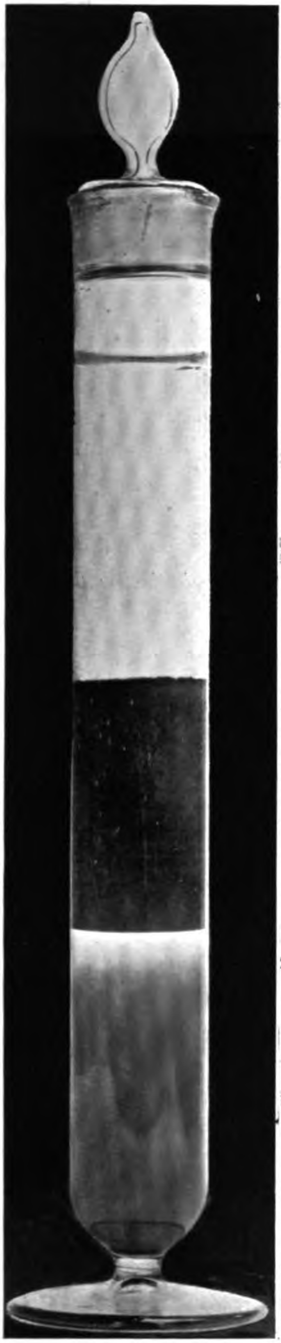


Fig. 1

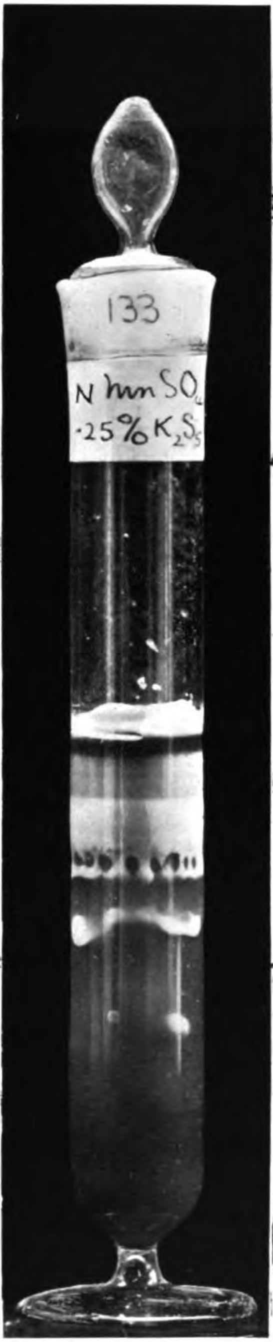


Fig. 2

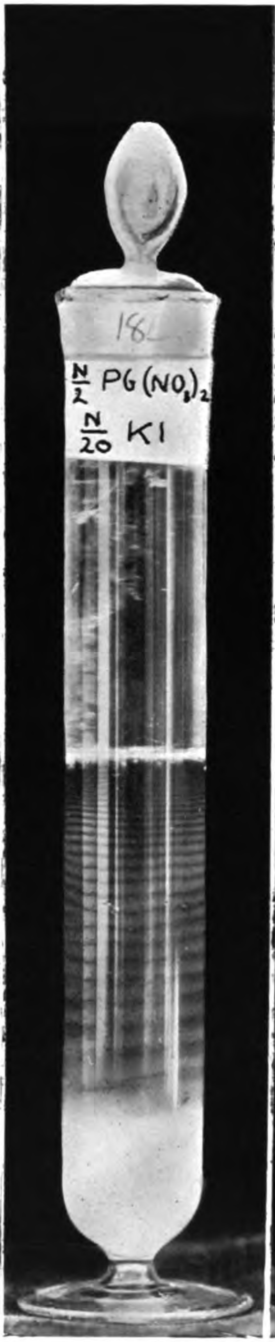


Fig. 3 *a*

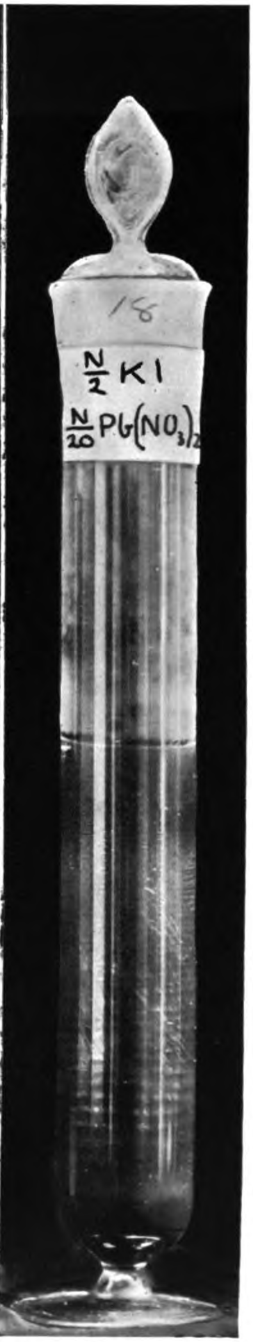


Fig. 3 *b*

reagent above the gel. In the case of a pair of reagents with different ratios *U/C*, the distances apart of the strata should turn upon which of the reagents is dissolved in the gel. This is illustrated by the tubes represented in Figs. 3 *a* and 3 *b* (Plate I). The former contained N/20 KI in 1 % agar with N/2 $Pb(NO_3)_2$ solution above, while in the latter tube the gel was made up with N/20 $Pb(NO_3)_2$ and treated with N/2 solution of KI.

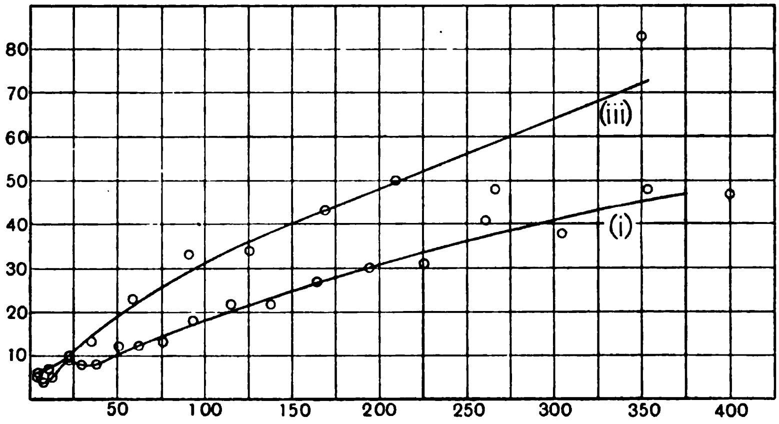


Fig. 5

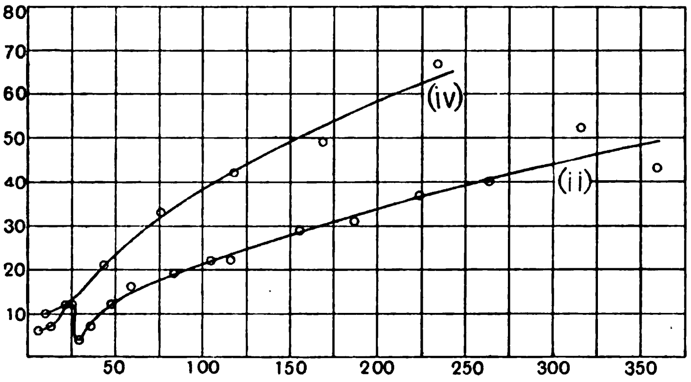


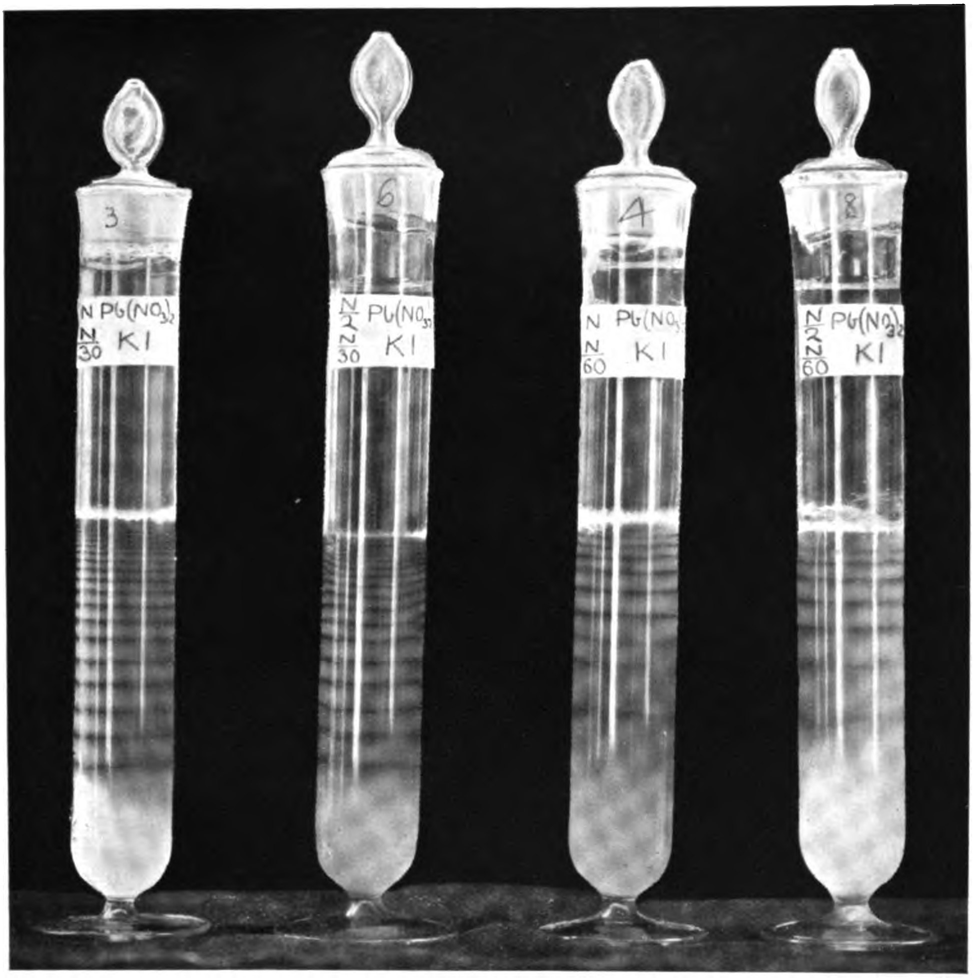
Fig. 6

Experiments with tubes, Fig. 4 (Plate II), containing (i) N/30 KI agar + N $Pb(NO_3)_2$, (ii) N/30 KI agar + N/2 $Pb(NO_3)_2$, (iii) N/60 KI agar + N $Pb(NO_3)_2$ and (iv) N/60 KI agar + N/2 $Pb(NO_3)_2$ show how, in this case, the distances apart of the layers are dependent rather upon the concentration of the solute in the gel than upon that of the reagent above. The curves, Figs. 5 and 6, were drawn freely through points obtained by plotting the distances apart of the strata as ordinates against the distances down the tube as abscissae. The lower curves, which correspond to nos. (i) and (ii), are too nearly coincident to be drawn on the same diagram. There is a little more distinction between the upper curves. As the strata take some days to form, the irregularities of the points may be due to variations of temperature. In many cases, however, the formation of the laminae is more regular.

Since Lagergren [1899] had observed a negative adsorption in the solutions of certain salts, *e.g.* sodium chloride, it seemed desirable to ascertain whether stratification could be obtained in gels impregnated with such a substance. Accordingly gels were prepared containing sodium chloride of various concentrations, and upon these were poured solutions of silver nitrate. The character of precipitate produced was quite different from that resulting with other solutes, being merely a diffuse cloudiness without any definite margin indicating the limit of the diffusion, which also took place much more quickly than had hitherto been observed. In no instance was any stratification noticed. However tubes prepared with silver nitrate of similar concentrations in the gel, and sodium chloride above, also showed no layers. This precipitate however was bounded by a definite margin, as in other cases, until the gel had become nearly exhausted of solute, when a diffuse precipitate rapidly extended to the bottoms of the tubes.

The differences in the character of the AgCl precipitate, according to which reagent is contained in the gel, recall similar divergencies well-known in volumetric analysis when decinormal solutions of silver nitrate and sodium chloride are mixed. It may be that the two varieties of milky and curdy precipitates are due to their formation in solutions having, in the one case negative, and in the other, positive adsorption values, which would affect the concentrations of the solutions in the neighbourhood of the precipitates. This would seem to be supported by the fact that many metallic chlorides give a curdy precipitate when silver nitrate is added to them in dilute solution.

Since gels containing sodium chloride could not be made to produce stratification in straight tubes, experiments were made in U-tubes containing pure 1 % agar, the limbs of which were filled up with solutions of sodium chloride and silver nitrate respectively. Contrary to expectation, stratification was produced whichever reagent was hypertonic, although the same differences in the character of the precipitates were noticed. When hypertonic sodium chloride diffused into silver nitrate a dense curdy column of silver chloride was produced, separated, at intervals of about a centimetre, by clear cut



i ii iii iv

Fig. 4

interspaces about a millimetre thick of transparent gel. Under the reverse condition only a slight diffuse cloudiness was formed on the first meeting of the solutions. This extended along the tube for about 12 or 15 mm., gradually filling the area of the tube and becoming a little denser. As further diffusion took place the first very light precipitate gradually disappeared, as if it had been swept up towards the denser portion by the diffusing reagent. This happened in every case, and, as other instances of the motion of visible particles in the gel have been observed, the effect may be due to this cause. After a time thin discs of denser precipitate appeared to be forming in the diffuse column. This was attributed to the backward diffusion of the sodium chloride after the strength of the silver solution had been diminished both by precipitation and adsorption. Obviously this explanation was not of general application. Consequently U-tubes of agar gel were treated with N/10 $AgNO_3$ and N/100 NaCl. After the production of the first light streaks, a great number of thin discs began to make their appearance at about 4 mm. apart, the distance rapidly decreasing to about 1 mm. On account of the low concentration of sodium chloride employed the discs could not be explained by alternate diffusion of the reagents. It seemed to follow, either that sodium chloride is positively adsorbed by silver chloride, or that the lamination resulted from the action of negative adsorption.

Attempts were then made to determine the nature of the adsorption of sodium chloride from solution by precipitated silver chloride. 100 cc. of decinormal NaCl solution were poured into well-stoppered bottles and to these were added 50 cc. of decinormal silver nitrate, thus producing a milky precipitate of silver chloride in a N/30 solution of sodium chloride. The bottles were allowed to stand 48 hours with occasional shaking, the clear supernatant liquid was then drawn off, 50 cc. at a time, in a pipette provided with a stopcock to prevent agitation of the precipitate. Titrations with decinormal silver nitrate indicated an adsorption of – 0·06 millimols per gram of AgCl in thirtieth normal solution, and of – 0·04 millimols in 0·0094 normal solution at 14°[1]. Lagergren's figures for charcoal in 0·98 normal concentration were – 0·09 millimols per gram at 0°. These experiments indicated that the stratification in sodium chloride gels must be explained by negative adsorption.

Further observations with U-tubes showed the occurrence of three types of stratification: (i) separate discs with no remaining diffuse precipitate, as previously described in the case of N/100 NaCl, (ii) a number of denser discs of chloride with extensions of very diffuse precipitate in the direction of diffusion and clear interspaces, and (iii) a column of diffuse precipitate interspersed, at intervals of about 2 mm., with very thin laminae of much denser precipitate. These effects may possibly be explained as follows. Owing to the negative adsorption of sodium chloride, the region of gel near the precipitate becomes gradually weaker in solute, while the next layer increases in strength. The excess of sodium chloride in this zone will tend to diffuse away, but will be hindered by the momentum of the particles flowing down the tube towards the precipitate. If the gel is very dilute, the region next the precipitate may not retain sufficient solute to produce a precipitate and the silver salt will be able to diffuse further until it enters the zone of stronger sodium chloride. This would give rise to a stratification of the first type. A gel which had been treated with a somewhat more concentrated solute might yield a precipitate gradually becoming less dense to zero before the diffusing silver salt reached the stronger layer of sodium chloride. This would correspond to type two. If the sodium chloride were again a little stronger, the clear spaces might not be produced at all, as in the third type.

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[1] 50 cc. supernatant liquid required 16·9, 16·9, 16·85, 4·7, 4·7 and 4·8 cc. N/10 $AgNO_3$ respectively.

Experiments in hand, which have now been interrupted, indicate that the remarkable influences of different gels upon the results obtained may be explained by v. Weimarn's theory on the assumption of protective action. For example lead iodide gives stratifications of gelatinous precipitate with difficulty in 5 % gelatin, while coarse precipitates in beautiful laminae occur in 1 % agar, and merely large aggregates in silicic acid gels. These results can be modified by varying the solubility of the precipitate, the concentration of the solute, or in other ways.

The following table (p. 175) records the positions of the strata formed in a number of experiments, measured in millimetres from the top of the gel. In many cases there were previous strata which were too close together for their positions to be read on a scale. The gel was 1 % agar except in the first case and the substance first mentioned was dissolved in the gel.

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Reference No.																							
9*a*	Silver dichromate in 10 % gelatin	3·6	7·6	11·75	16·2	21·2	26·25	32·1	38·1	44·25	53·1												
13	N/30 $CaCl_2$ + conc. Na_2CO_3	26·0	29·0	34·5	43·0	54·0																	
14	N/20 $CaCl_2$ + conc. Na_2CO_3	22·0	23·5	26·75	30·0																		
19	N/20 Na_2CO_3 + dil. $CaCl_2$	20·5	22·5	25·0	28·0	32·0	35·5																
20	N/20 Na_2CO_3 + dil. $CaCl_2$	20·5	22·5	25·5	28·5	32·5	36·0																
21	N/20 Na_2CO_3 N/20 NaCl } + dil. $CaCl_2$	20·5	22·5	24·5	—	33·0	35·5																
22	N/20 Na_2CO_3 N/20 NaCl } + dil. $CaCl_2$	20·5	23·0	—	—	31·5	35·5																
38	dil. $AgNO_3$ + conc. $K_2Cr_2O_7$	14·0	16·3	19·5	23·7	29·0	35·5	44·0															
50	N/20 Na_2CO_3 + conc. $CaCl_2$	24·5	28·5	32·5																			
51	N/20 Na_2CO_3 N/20 NaCl } + conc. $CaCl_2$	24·0	28·5	33·5	38·0																		
79	N/60 $K_2Cr_2O_7$ + N/5 Pb $(NO_3)_2$	56·1	57·2	58·5	59·8	61·0																	
119	N/50 KI + N Pb $(NO_3)_2$	0·4	1·0	1·9	2·9	4·0	5·2	6·5	8·1	9·9	12·0	14·5	17·1	20·2	23·4	27·0	30·9	35·0	39·4	44·1			
120	N/20 KI + N Pb $(NO_3)_2$	5·0	5·7	6·5	7·4	8·4	9·5	10·7	12·0	13·4	15·1	17·0	19·1	21·4	23·8	26·2	29·5	33·4	37·7	42·3			
129	N/50 KI + N/5 Pb $(NO_3)_2$	0·5	1·1	1·9	3·0	4·2	5·6	7·2	9·1	11·2	13·7	16·6	20·3	24·6	shorter tube								
139	N/50 KI + NPb $(NO_3)_2$	0·6	1·3	2·3	3·4	4·8	6·2	7·8	9·4	11·2	13·6	15·7	18·3	21·8	25·2	28·8	32·5	36·5					
140	N/50 KI + N/2 Pb $(NO_3)_2$	1·3	2·4	3·7	5·0	6·8	8·8	11·2	13·5	16·3	19·7	23·0	26·7	30·8	35·0								
130	N/20 $MnSO_4$ + 5 % K_2S_x	2·7	3·8	4·5	5·7	6·8	7·9	9·1	10·1	11·3	13·0	14·3	15·9	16·8	17·9	18·9	20·0	23·0	27·0	32·4	40·3	51·2	
133	0·25 % K_2S_x + N $MnSO_4$	2·0	—	—	8·0	10·5	15·0	19·0	26·0	40·0													
134	0·09 % K_2S_x + N $MnSO_4$	7·0	11·0	16·0	24·0	33·0	45·0																
154	N/30 KI + N Pb $(NO_3)_2$	0·6	1·1	1·6	2·4	3·2	3·9	5·0	6·2	7·5	9·3	10·8	12·8	15·0	17·1	20·3	23·6	26·8	30·5	34·8	39·7		
155	N/30 KI + N Pb $(NO_3)_2$	0·5	0·9	1·4	2·3	3·1	3·9	5·1	6·3	7·6	9·4	11·6	13·8	16·5	19·5	22·6	26·7	30·5	35·3	40·0			
156	N/60 KI + N Pb $(NO_3)_2$	0·6	1·3	2·3	3·6	5·9	9·2	12·6	16·9	21·9	26·7	35·0											
157	N/60 KI + N Pb $(NO_3)_2$	0·4	1·3	2·8	4·9	7·6	10·5	13·6	17·8	24·2	31·6												
158	N/30 KI + N/2 Pb $(NO_3)_2$	0·6	1·3	2·5	2·9	3·6	4·8	6·4	8·3	10·5	12·7	15·6	18·7	22·4	26·4	31·6	35·9						
159	N/30 KI + N/2 Pb $(NO_3)_2$	0·9	1·6	2·3	3·3	4·2	5·7	7·0															
160	N/60 KI + N/2 Pb $(NO_3)_2$	1·0	2·2	4·3	7·6	11·8	16·7	23·4															
161	N/60 KI + N/2 Pb $(NO_3)_2$	0·9	2·7	4·3	7·2	11·2	15·8	21·3															
184	N/20 KI + N/2 Pb $(NO_3)_2$	3·4	4·0	4·8	5·6	6·3	7·2	8·1	9·0	10·2	11·6	13·1	14·9	16·8	18·8	21·6	23·8	25·2	27·9	30·7	33·8	37·1	41·8
186	N/20 Pb $(NO_3)_2$ + N/2 KI	18·4	18·9	19·5	20·1	20·7	21·3	22·0	22·7	23·4	24·1	24·8	25·5	26·3	27·2	28·1	29·1	30·2	31·3	32·4	33·5	34·7	35·8
														37·1	38·4	39·6	40·9	42·4	44·1	45·9	48·0	50·1	52·0

XVIII. A NEW COLOUR REACTION FOR "OXYCHOLESTEROL."

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(*Received March 20th, 1916.*)

In a previous communication dealing with the presence of "Oxycholesterol" in brain [1914], I have mentioned that this substance, when moistened with dimethyl sulphate (techn.) gives immediately a characteristic purple colour reaction, while under the same conditions cholesterol remains unaffected[1]. The latter assumes gradually, especially on warming, a bright raspberry-red colour. Under identical conditions pure dimethyl sulphate fails to react with either substance.

When the colouring matters were examined in chloroform solution, they were found to possess characteristic spectra.

An interesting reaction occurs in the presence of oxidising agents, of which ferric chloride is the most convenient for practical purposes. On addition of a drop of ferric chloride in glacial acetic acid solution, the bright raspberry-red colour of the cholesterol reaction changes into purple, while the purple of the "oxycholesterol" reaction changes into a brilliant emerald green. The latter shows a well-defined and characteristic absorption band in the red between λ 650–λ 630, which is identical with that obtained in the Lifschütz reaction [1908]. I found further that *pure* dimethyl sulphate in the presence of ferric chloride also gave the same emerald green colour with "oxycholesterol," while cholesterol under the same conditions gave no colour reaction even on prolonged warming. These reactions can be used to detect "oxycholesterol" in presence of cholesterol, and also to estimate its quantity spectrometrically.

The striking difference between the behaviour of technical and of pure dimethyl sulphate was next investigated. The active substance in technical dimethyl sulphate which reacts with "oxycholesterol" and with cholesterol in the absence of ferric chloride is evidently an impurity and my experiments tend to show that it is monomethyl sulphate. This probably owes its origin to partial hydrolysis of dimethyl sulphate in a moist atmosphere, and its formation explains the fact that even inactive *pure* dimethyl sulphate gradually becomes active on keeping under ordinary laboratory conditions.

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[1] This reaction has since been successfully applied by Lamb [1914] in a study of the influence of atmospheric oxygen on the oxidation of cholesterol under histological conditions.

The dimethyl sulphate reaction cannot serve for the distinction of animal and vegetable cholesterols, as I have found that sitosterol as well as "oxysitosterol" give colour reactions identical with those of cholesterol and "oxycholesterol" respectively.

EXPERIMENTAL.

The cholesterol used was prepared from human brain by the method of O. Rosenheim [1906]. "Oxycholesterol" was prepared from it by treatment with benzoyl peroxide in glacial acetic acid solution, according to Lifschütz [1913]. The spectroscopic observations were made with a Zeiss comparison spectroscope. The depths of the fluids examined were measured in a special absorption vessel, which allows measurements to 0·05 mm. A small electric arc lamp, at 20 cm. distance from the apparatus and screened by a plate of matted glass, served throughout the experiments as the source of light. The width of the spectroscope slit was 0·2 mm. in all observations.

I. *Reaction with technical dimethyl sulphate.*

(*a*) *Cholesterol.* On adding a few drops of technical dimethyl sulphate (Kahlbaum) to a solution of cholesterol in chloroform, the solution remains colourless for some minutes, but on slightly warming in a water-bath it gradually assumes a bright raspberry-red colour[1]. In sunlight, or in the light of the electric arc, it shows a blue fluorescence. Solutions of cholesterol in carbon tetrachloride, benzene, toluene or xylene give similar colour reactions, the colours tending to be more blue, less transparent and more fluorescent. Solutions in ether, alcohol, acetone, amyl acetate and amyl alcohol failed to give any reactions. The colour of the chloroform solution is discharged by the addition of water or alcohol. This fact distinguishes the reaction from that of Neuberg and Rauchwerger [1904] with methylfurfural, which is carried out in alcoholic solution. This also gives a raspberry-red solution, but shows a different spectrum.

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[1] This reaction was discovered accidentally by Dr O. Rosenheim when carrying out experiments on the methylation of cholesterol.

On adding a drop of ferric chloride solution (5 % ferric chloride in glacial acetic acid), the colour is intensified and changes into a deep purple. The reaction is distinguished by great permanency and was observed to persist for more than a week.

For the spectroscopic examination 0·2 cc. of Kahlbaum's dimethyl sulphate (techn.) was added to 2 cc. of a 5 % cholesterol solution in dry chloroform. A deep raspberry-red colour developed on slight warming which showed two characteristic bands, one between *D* and *E* and the other near *F*. The following observations in the absorption vessel were made:

Depth of fluid	
5·0 mm.	only red light transmitted.
3·0 ,,	one dense band in yellow and green from $\lambda 595$–$\lambda 460$.
1·5 ,,	one dense band in yellow and green from $\lambda 585$–$\lambda 485$.
1·0 ,,	two bands from $\lambda 575$–$\lambda 525$ and from $\lambda 505$–$\lambda 488$.
0·7 ,,	,, from $\lambda 565$–$\lambda 535$ and from $\lambda 505$–$\lambda 485$.
0·5 ,,	,, from $\lambda 560$–$\lambda 550$ and from $\lambda 500$–$\lambda 490$.
0·4 ,,	one band at $\lambda 500$.

The appearance of the two characteristic bands is shown in Fig. 1 (Plate III).

When one drop of the ferric chloride reagent was added, the solution became so intensely coloured that accurate observations were impossible without dilution. The experiment was therefore repeated using a 1 % solution of cholesterol. On addition of ferric chloride the solution became purple and transmitted only red light when examined in 5 mm. thickness.

The following observations were made:

Depth of fluid	
5·0 mm.	only red light transmitted.
1·3 ,,	blue light also is transmitted.
1·0 ,,	two bands from $\lambda 540$–$\lambda 530$ and from $\lambda 510$–$\lambda 420$.
0·5 ,,	one band only from $\lambda 505$–$\lambda 480$.

The reaction is of approximately the same sensitiveness as Liebermann's cholesterol reaction, the limit being reached when 2 cc. of the solution contained 0·02 mg. of cholesterol.

(*b*) "*Oxycholesterol.*" A solution of this substance in chloroform gives on the addition of a few drops of technical dimethyl sulphate an immediate purple colour without warming (stage 1). A drop of the ferric chloride reagent added changes this at once into a greenish blue which gradually turns into an emerald green (stage 2) showing a green fluorescence. The

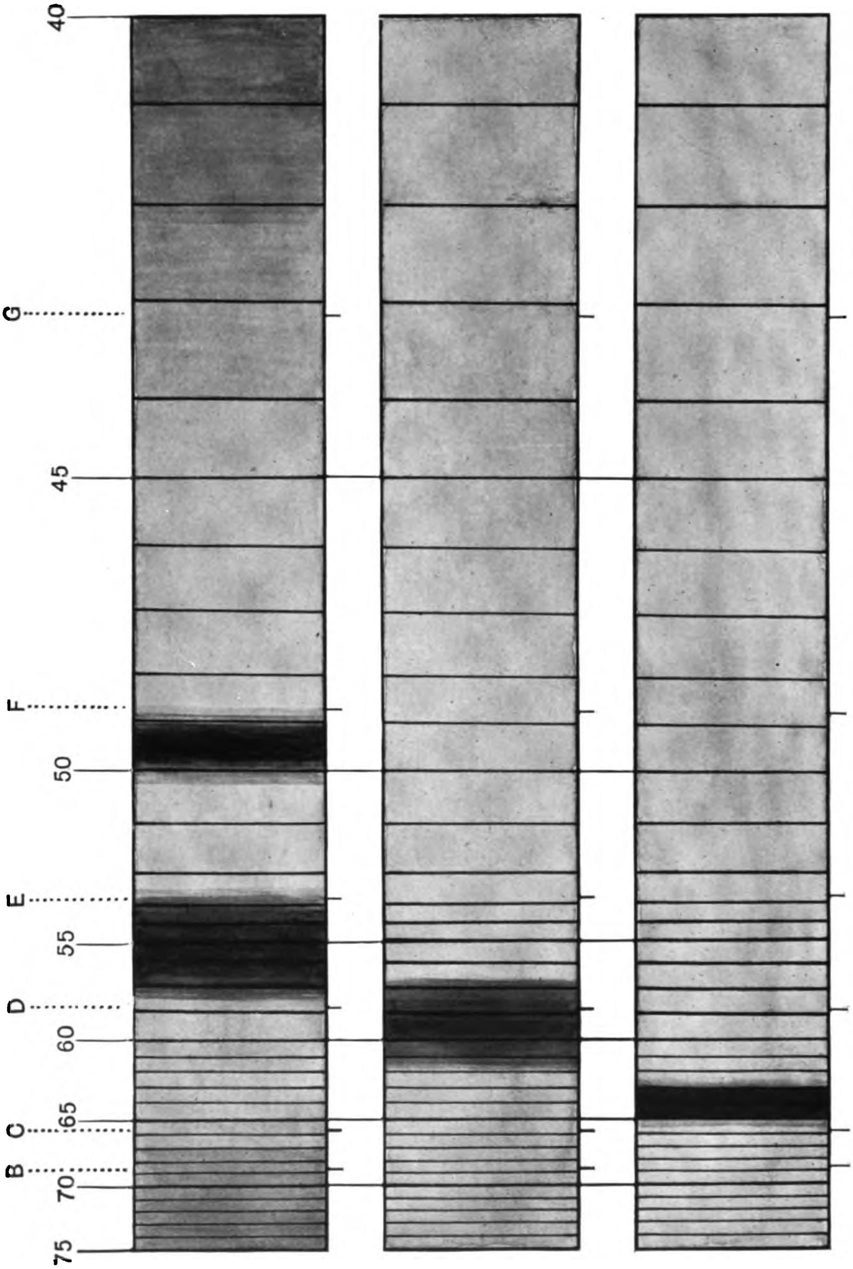

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Spectrum of Cholesterol Reaction, 0·02 g. in 2 cc. chloroform, thickness of layer: 1 mm.
Fig. 2. Spectrum of "Oxycholesterol" Reaction, 1st stage 0·02 g. in 2 cc. chloroform, thickness of layer: 0·5 mm.
Fig. 3. Spectrum of "Oxycholesterol" Reaction, 2nd stage, 0·02 g. in 2 cc. chloroform, thickness of layer: 0·5 mm.

green colour is discharged by water, acetone, alcohol or amyl alcohol, but not by glacial acetic acid, chloroform or benzene. Glacial acetic acid appears to be the most suitable diluent, the solution remaining transparent, whilst chloroform or benzene gives rise to some turbidity.

For spectroscopic examination 0·2 cc. of dimethyl sulphate (techn.) was added to 2 cc. of a 1 % "oxycholesterol" solution. Observations could only be made in thicknesses from 1·8 mm. downwards. All the spectral colours pass through the solution and there is only one band near the *D* line which shows best in a thickness of from 0·5 to 0·3 mm.

Depth of fluid	
1·8 mm.	one deep broad band from λ 620 to λ 520.
0·5 ,,	one broad band; blue, green and red light transmitted brilliantly.
0·3 ,,	one distinct band in the yellow, from λ 600—λ 580.
0·15 ,,	one faint band at *D* line—at λ 590.

The characteristic band of the first stage is shown in Fig. 2 (Plate III). On addition of two drops of glacial acetic acid and one drop of the ferric chloride reagent, the solution becomes greenish blue at once and rapidly reaches the second stage of emerald green. This also shows one band which is however in the red. The solution had to be diluted with an equal volume of acetic acid before satisfactory measurements could be made. The following observations were made with a freshly prepared solution containing only 0·5 % of "oxycholesterol."

Depth of fluid	
10·0 mm.	absorption of red end of spectrum as far as λ 560.
6·5 ,,	red light transmitted; blue and green come through undiminished.
5·0 ,,	one band from λ 670–λ 630.
4·0 ,,	one sharply defined band from λ 660–λ 630.
3·0 ,,	,, ,, ,, ,,
1·2 ,,	one band still shows faintly at λ 650.

Fig. 3 (Plate III) shows the typical band of the second stage in a 1·0 % solution of 0·5 mm. thickness.

The sensitiveness of the "oxycholesterol" reaction is somewhat less than that of the cholesterol reaction under the same conditions. The limit is reached when 0·2 mg. of "oxycholesterol" is contained in 2 cc. of chloroform; but on the other hand, the colour once produced in a stronger solution stands an amount of dilution which is much greater than the above limit.

The formation of "oxycholesterol" from cholesterol may be very well shown by means of the above reaction, when a small quantity of cholesterol is boiled in acetic acid solution with benzoyl peroxide, according to Lifschütz [1908]. On the addition of a few drops of dimethyl sulphate (techn.) a brilliant blue colour develops at once, which changes into green on standing, or immediately after the addition of a drop of ferric chloride solution. The green solution shows the single band in the red of the "oxycholesterol" reaction in the typical manner.

II. *Reaction with pure dimethyl sulphate.*

(*a*) *Cholesterol.* Pure dimethyl sulphate does not give rise to any colour reaction with either dry cholesterol or its solutions in chloroform or other solvents. The solutions remain colourless also on warming in a water-bath or after the addition of a drop of the ferric chloride reagent.

(*b*) "*Oxycholesterol.*" Strong "oxycholesterol" solutions in chloroform show, after the addition of pure dimethyl sulphate and warming in the water-bath, a slight purple colour which changes after the addition of ferric chloride into the typical green colour, showing the characteristic single band in the red. Even dilute "oxycholesterol" solutions which remain colourless with pure dimethyl sulphate, still turn green after the addition of ferric chloride, and show the "oxycholesterol" band in the red.

III. *The nature of the active substance in technical dimethyl sulphate.*

The fact that pure dimethyl sulphate fails to react with cholesterol makes it evident that the active substance in the technical product is of the nature of an impurity. An attempt was made to isolate this impurity by fractional distillation, but it was found that all the fractions as well as the residue in the distilling flask gave equally strong reactions. It was next observed that inactive pure dimethyl sulphate became active when it was simply heated to boiling in a test tube. At the same time it became strongly acid to Congo paper, while it was previously neutral. It was further noticed that a sample of inactive pure dimethyl sulphate, which had been kept for a considerable time in a bottle under ordinary laboratory conditions, had become active and had at the same time acquired an acid reaction. A control sample, kept in a sealed tube for the same length of time, had remained inactive. Further, it was found that all the samples of active technical dimethyl sulphate examined possessed an acid reaction to Congo paper.

It is well known that dimethyl sulphate is partially decomposed into methyl alcohol and monomethyl sulphate on heating to the boiling point, as well as by partial hydrolysis, and monomethyl sulphate may be further hydrolysed into methyl alcohol and sulphuric acid. The latter cannot be considered as the active substance since the addition of small quantities of concentrated sulphuric acid to pure dimethyl sulphate failed to activate it. Further, sulphuric acid gives rise to the well-known Salkowski's reaction, from which the dimethyl sulphate reaction is characteristically different, both in its appearance and in its behaviour towards ferric chloride.

It seemed therefore most likely that the acid monomethyl sulphate was responsible for the reaction. Since this substance forms a barium salt which is insoluble in chloroform, an indirect proof for this assumption may be obtained. A sample of technical dimethyl sulphate, dissolved in chloroform, was treated with solid barium carbonate until the effervescence had ceased. The filtered solution was found to be inactive and left, on careful evaporation of the chloroform, an oil which reacted neutral towards Congo paper. The inactive dimethyl sulphate thus prepared was easily activated again by boiling it for a short time in a test tube. Since the preparation of pure monomethyl sulphate from its barium salt is a matter of some difficulty, I have refrained from further testing this question.

There remained still the possibility that the activity of technical dimethyl sulphate might be due to traces of formaldehyde or formic acid, formed by gradual oxidation of methyl alcohol which is set free by hydrolysis. Formaldehyde and formic acid were indeed found to give rise to colour reactions with cholesterol under certain conditions, which need not be described here. But pure dimethyl sulphate does not produce any colour reaction in the presence of a trace of formaldehyde or formic acid, and there is therefore no reason to ascribe to either of them any share in the reaction of technical dimethyl sulphate with cholesterol.

The reaction of cholesterol with monomethyl sulphate may be used conversely for testing the purity of dimethyl sulphate preparations. Since technical dimethyl sulphate is probably manufactured by distillation under ordinary pressure, most of the samples contain monomethyl sulphate in varying amounts, whilst a distillation *in vacuo* furnishes a pure product which does not react with cholesterol.

It may be mentioned that diethyl sulphate behaves in exactly the same way. The pure substance does not react with cholesterol, but with "oxycholesterol" gives rise to a typical green reaction with ferric chloride, showing the characteristic absorption band between λ 650 and λ 630. By heating the pure product to boiling in a test tube it also became active towards cholesterol and then behaved just like technical dimethyl sulphate.

Cholesterol esters give the dimethyl sulphate reaction in a less degree but vegetable cholesterols (phytosterols) behave exactly like cholesterol. A phytosterol prepared from hemp-seed and sitosterol from wheat embryos (prepared according to Ritter [1902]) were tested. The corresponding "oxysitosterol" reaction also was spectroscopically indistinguishable from that of "oxycholesterol."

The relationship of cholesterol to the terpenes suggested an examination of the behaviour of some of these substances towards dimethyl sulphate. It was found that borneol, camphor and abietinic acid (prepared from colophony), as well as cholalic acid, all give rise to colour reactions with technical dimethyl sulphate, the description of which is outside the scope of this communication. But none of these substances, when subjected to oxidation with benzoyl peroxide, as in the preparation of "oxycholesterol," gave rise to the typical "oxycholesterol" reaction with dimethyl sulphate.

SUMMARY.

1. A colour reaction of cholesterol with technical dimethyl sulphate is described, the spectrum showing two bands, from λ 560–λ 550 and from λ 500–λ 490.

2. This reaction is not given by *pure* dimethyl sulphate and appears to be due to the presence of monomethyl sulphate.

3. "Oxycholesterol" gives a purple colour reaction with technical dimethyl sulphate showing an absorption band in the yellow (λ 600–λ 580).

4. Pure or technical dimethyl sulphate, after the addition of ferric chloride, gives a typical green colour reaction with "oxycholesterol," showing a well-defined absorption band in the red between λ 650 and λ 630.

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XIX. THE DECOMPOSITION OF LACTIC ACID BY KILLED YEAST.

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According to the theory that the first stages both of anaerobic respiration and of alcoholic fermentation consist of a series of oxidations and reductions at the expense of water [Palladin, 1912], it must be concluded that amongst the first products of decomposition of glucose must be placed organic acids. These acids must undergo further anaerobic oxidation in which reductase participates. Palladin and Mlle Lovchinovskaya [1914] therefore investigated the action of a hydrogen acceptor (methylene blue) on the decomposition of some organic acids—supposed to be present among the intermediate products of the decomposition of glucose—by killed yeast. Of the acids investigated, gluconic and lactic caused the evolution of a greater quantity of carbon dioxide in the presence of methylene blue, than in its absence. The action of reductase consists in removing the hydrogen from one substance and transferring it to another. The substance which yields the hydrogen is called the oxidisable substance, or reductor ($R.H_2$), whereas the substance receiving the hydrogen is called the hydrogen acceptor, or oxidator (A):

$$R.H_2 + A = R + A.H_2.$$

If we take the case in which reduction takes place with the participation of water, the hydrogen of the water will join the hydrogen acceptor, and the oxygen will join the oxidisable substance and either remove from it hydrogen with the formation of water, or produce some other oxidising reaction.

The oxidations produced by reductase by means of removing the hydrogen may be accompanied by the evolution of carbon dioxide. Thus, Bredig and Sommer [1910] have shown that formic acid is decomposed into carbon dioxide and hydrogen in the presence of a catalyst and methylene blue:

$$HCO_2H + M = CO_2 + M.H_2.$$

The carbon dioxide produced in the decomposition of gluconic and lactic acids also owes its production to reductase. Having put off for some time the detailed investigation of gluconic acid, we turned to the study of the decomposition of lactic acid. The results of this research form the contents of the present paper.

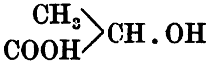
We started from the supposition that in the presence of the hydrogen acceptor (A) lactic acid first produces pyruvic acid under the influence of reductase:

$$(1)\quad CH_3 . CH(OH) . COOH + A = CH_3 . CO . COOH + A . H_2 .$$

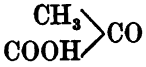
The pyruvic acid formed is then decomposed into carbon dioxide and acetaldehyde under the influence of carboxylase:

$$(2)\quad CH_3 . CO . COOH = CH_3 . CHO + CO_2 .$$

Beilstein and Wiegand [1885], thinking lactic acid to be a secondary alcohol and pyruvic acid to be the ketone corresponding to it,



$$\left.\begin{matrix}CH_3\\COOH\end{matrix}\right\rangle CH . OH$$



$$\left.\begin{matrix}CH_3\\COOH\end{matrix}\right\rangle CO$$

obtained pyruvic acid by oxidising calcium lactate with potassium manganate. Wieland [1912] subsequently found that when palladium black acts on lactic acid, pyruvic acid is also produced. We worked with hefanol and zymin which were considerably weakened by rather long preservation. During the first half of our work we used methylene blue as a hydrogen acceptor, and in the second half acetaldehyde *in statu nascendi.*

I. THE RATIO OF THE QUANTITY OF ACETALDEHYDE FORMED TO THE QUANTITY OF CARBON DIOXIDE EVOLVED IN PRESENCE OF METHYLENE BLUE.

In all the experiments of this work the lactic acid was neutralised by caustic potash. The reaction therefore follows the equations:

$$(1)\quad 2CH_3 . CH(OH) . COOK + 2M = 2CH_3 . CO . COOK + 2MH_2 ,$$

$$(2)\quad 2CH_3 . CO . COOK + H_2O = 2CH_3 . CHO + CO_3K_2 + CO_2 .$$

As is seen, part of the carbon dioxide remains combined in the solution. In order to produce it in a free state, 10 drops of 3 % sulphuric acid were added after the termination of the experiment. In that case:

$$\frac{CO_2}{CH_3CHO} = \frac{44}{44} = 1.$$

The experiments were conducted in round-bottomed flasks with long necks, with a capacity of 50 cc. The flasks were closed with rubber corks with three holes. Through one hole a tube passed to the bottom, through the second, a short tube. Both tubes carried at the top india-rubber tubes which were tightly closed during the experiment by means of stoppers. Into the third opening a funnel with a tap was fitted, through which the sulphuric acid was poured. During the experiment the flasks were shaken up in a special shaking apparatus. On this account even a partial decolorisation of the methylene blue was never observed. After the addition of sulphuric acid a current of air was conducted through the flask and then through a Pettenkofer's tube.

As qualitative reactions for acetaldehyde, the reaction with fuchsin-sulphurous acid and the reaction of Rimini were used. The quantitative determination of acetaldehyde was carried out by distillation into a flask cooled by snow. In the distillate the aldehyde was determined by adding bisulphite and titrating the excess by iodine according to Ripper's method.

Exp. 1. Three flasks. In each 7 g. of hefanol and 25 cc. of 0·5 % methylene blue. In the first and second 25 cc. of 2 % potassium lactate. In the third, 25 cc. of water. The duration of the experiment was 3 h. 20 m. Temperature 17–18°.

Results: No. 1. 75·0 mg. CO_2.
„ 2. 31·3 „ acetaldehyde.
„ 3. 40·6 „ CO_2.

After subtracting from 75·0 the carbon dioxide of auto-fermentation, we have 34·4. Therefore,

$$\frac{CO_2}{CH_3.CHO}=\frac{34\cdot4}{31\cdot3}=1\cdot1.$$

Exp. 2. Repetition of the preceding. Duration 6 hrs. Temperature 17–18°.

Results: No. 1. 94·6 mg. CO_2.
„ 2. 46·2 „ acetaldehyde.
„ 3. 41·2 „ CO_2.

$$94\cdot6 - 41\cdot2 = 53\cdot4,$$

$$\frac{CO_2}{CH_3.CHO}=\frac{53\cdot4}{46\cdot2}=1\cdot16.$$

Exp. 3. Repetition of preceding. Duration 7 hrs. Temperature 17–18°.

Results: No. 1. 99·1 mg. CO_2.
„ 2. 49·0 „ acetaldehyde.
„ 3. 48·2 „ CO_2.

$$99\cdot1 - 48\cdot2 = 50\cdot9,$$

$$\frac{CO_2}{CH_3.CHO}=\frac{50\cdot9}{49\cdot0}=1\cdot04.$$

Thus, in all the three experiments the ratio CO_2/CH_3CHO is, according to the theory, nearly equal to one, if we hold that in the presence of lactic acid auto-fermentation continued with the same energy as in the control experiment.

The question arises, why was not the acetaldehyde further reduced to ethyl alcohol? It is quite possible that the presence of a large quantity of the hydrogen acceptor, which largely takes away the hydrogen, prevents this reaction. It may be expressed in the form of a general rule that *in the presence of a large quantity of the hydrogen acceptor the formation of alcohol is impossible.* If applied in the necessary manner, the hydrogen acceptor can be made to interrupt the alcoholic fermentation at the stage of acetaldehyde.

The experiments described above have already appeared in Russian in a paper by Palladin, Sabinin and Lovchinovskaya [1915] published on 1st May, 1915. In September there appeared a paper by Harden and Norris [1915], who, not knowing of our work, had also discovered that lactic acid is decomposed by dried yeast with the help of reductase and carboxylase, and that as a result acetaldehyde is produced. In this work there is mentioned the very interesting fact that only a small part of the oxidised lactic acid is converted into acetaldehyde. As Harden and Norris did not find any pyruvic acid, they suggested that possibly only part of the lactic acid was converted into pyruvic acid, the greater part oxidising otherwise. The following facts speak in favour of such a view: Palladin, Lovchinovskaya and Alexeieff [1915] discovered that methylene blue interrupts the decomposition of pyruvic acid by zymin and wheat germs. Further, according to the equation

$$2CH_3 . CO . COOK + H_2O = 2CH_3 . CHO + CO_2 + K_2CO_3,$$

it was to be expected that after the end of the experiment the quantity of carbon dioxide in the form of carbonate of potassium would be equal to the quantity of gaseous carbon dioxide evolved.

In reality it was different. In the control portion the combined carbon dioxide was less by 62 %, and in the experimental portion by 82 %, as is seen from the following experiment quoted from their work.

Exp. iv. Three portions of 5 g. of old zymin. 1. 50 cc. of 1 % pyruvic acid neutralised by caustic potash. Air current. 2. 50 cc. of 1 % pyruvic acid neutralised by caustic potash; 1·5 g. methylene blue. Hydrogen current. 3. 50 cc. water, 1·5 g. methylene blue. Hydrogen current. After 24 h. 10 m. the experiment was stopped. There was no decolorisation. Then 10 cc. 5 % H_2SO_4 were added to 1 and the same quantity to 2. 2 cc. of toluene in each flask. Temperature 14·5–15°.

	1 1 % potassium pyruvate. Air current		2 1 % potassium pyruvate. Methylene blue. Hydrogen current		3 Water. Methylene blue. Hydrogen current	
Time	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.
2 hrs.	32·2	16·1	22·2	11·1	4·6	2·3
2 ,,	25·8	12·9	14·2	7·1	4·2	2·1
20 ,,	41·1	2·0	38·7	1·9	7·0	0·3
24 ,,	99·1	—	75·1(−24%)	—	15·8	—
			H_2SO_4 added.			
3 hrs. 35 m.	38·6(−62%)	—	13·2(−82%)	—	—	—
Total	137·7	—	88·3	—	—	—

It is to be concluded from the evidence given in such experiments that parallel to the decomposition of both the pyruvic and the lactic acids, the formation of some acid or acids decomposing CO_3K_2 with the evolution of carbon dioxide takes place. In presence of methylene blue a greater quantity of these acids is formed, than in the control portions. Still less combined carbon dioxide is produced by decomposing lactic acid by killed yeast in the presence of methylene blue, as was shown in the paper by Palladin, Sabinin and Lovchinovskaya [1915] mentioned above. Lvoff [1913] discovered in our laboratory that in the process of glucose fermentation for each atom of hydrogen removed with the help of methylene blue, the production both of alcohol and of carbon dioxide is diminished by one molecule. This led Lovchinovskaya to try to find some similar laws for lactic acid.

According to the equations:

(1) $2CH_3 . CHOH . COOK + 2M = 2CH_3 . CO . COOK + 2MH_2$,

(2) $2CH_3 . CO . COOK + H_2O = 2CH_3COH + CO_3K_2 + CO_2$,

and taking into account that the molecular weight of methylene blue, $C_{16}H_{18}N_3SCl + 3H_2O$, is 373·5, it follows that the ratio

$$\frac{CO_2}{2M} = \frac{44}{747{\cdot}0}.$$

It follows from this, that for 1·5 g. of methylene blue before its complete decolorisation in an atmosphere of hydrogen there must be evolved 88·3 mg. of carbon dioxide. Experiments produced greater quantities.

Exp. 4. Three portions of 10 g. of hefanol. 1. 50 cc. 2 % lactic acid neutralised by caustic potash, and 1·5 g. methylene blue. Hydrogen current. 2. 50 cc. 2 % lactic acid neutralised by caustic potash. Air current. 3. 50 cc. of water. Air current. To each flask 2 cc. of toluene were added. After decolorisation of 1, the experiment was stopped. Temperature 16°.

Time	Potassium lactate and methylene blue. Hydrogen current		Potassium lactate. Air current		Water. Air current	
	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.
4 h. 45 m.	78·4	18·4	31·0	7·3	26·0	6·1
23 hrs.	177·6	7·7	63·6	2·7	53·0	2·3
24 h. 45 m.	256·0	—	94·6	—	79·0	—

256 − 94·6 = 161·4 instead of 88·3.

Similar experiments gave the following figures:

	161·4
	154·2
	139·6
	148·6
	108·8
	147·7
Average:	143·4

If we suppose that all the CO_2 had been evolved 88·3 × 2 = 176·6 mg. of CO_2 would have been obtained. In reality, the average was found to be 143·4, *i.e.* 81·2 %. Therefore, a small part of the carbon dioxide must have remained in a combined state, and this was confirmed by experiment.

Exp. 5. One portion of 11·5 g. dried yeast (Lebedeff); 50 cc. 1 % lactic acid neutralised by caustic potash; 1·5 g. methylene blue; 2 cc. of toluene. The experiment was stopped after 44 h. 20 m. There was no decolorisation. Afterwards 10 cc. 5 % H_2SO_4 were added. Temperature 14° C.

Duration of the experiment	Potassium lactate and methylene blue. Hydrogen current	
	CO_2 mg.	CO_2 in 1 h.
2 h. 30 m.	12·2	4·9
16 h. 10 m.	95·1	5·8
2 h. 40 m.	46·8	17·5
23 hrs.	58·0	2·5
44 h. 20 m.	212·1	—
Addition of H_2SO_4.		
24 hrs.	19·2	—
Total	231·3	—

Consequently, there appeared to be only 9 % of combined carbon dioxide.

II. THE RATIO OF THE QUANTITY OF CARBON DIOXIDE EVOLVED TO THE QUANTITY OF OXYGEN ABSORBED IN THE PRESENCE OF METHYLENE BLUE.

The experiments in this part of our work were undertaken on the basis of the following equations:

$$(1)\quad 2CH_3 . CHOH . COOK + 2M = 2CH_3 . CO . COOK + 2MH_2,$$

$$(2)\quad 2MH_2 + O_2 = 2M + 2H_2O,$$

$$(3)\quad 2CH_3 . CO . COOK + H_2O = 2CH_3 . CHO + CO_3K_2 + CO_2,$$

from which it follows that

$$\frac{CO_2}{O_2} = 1.$$

But the experiments of Lovchinovskaya proved that not merely half of the carbon dioxide is evolved, according to the equation, but a considerably greater amount. Therefore, the ratio CO_2/O_2 ought to be much larger than one. Nevertheless, it proved to be equal to one. This shows that, besides oxidising the reduced methylene blue, the oxygen enters into some other oxidation. In the first experiments fermentation took place at the temperature of the room in flat conical flasks similar to those used in cultivating diphtheria. The flasks were sealed by mercury, with two tubes—sealed by mercury—for removing the gas. During the experiment the flasks were shaken once or twice. This proved to be insufficient, the methylene blue having become partly decolorised at the end of the experiment, and high coefficients having been produced.

Exp. 6. Two flasks. In each 2 g. of hefanol and 30 cc. 1 % potassium lactate. In addition, in the second 3 cc. 0·5 % methylene blue, and in the first 30 cc. water. Duration of the experiment 5·5 hours. In all the experiments the gas was analysed in the Polovtzeff-Richter apparatus.

Flask No. 1	Flask No. 2
CO_2 = 3·5 %	CO_2 = 1·41 %
O_2 = 19·3 %	O_2 = 19·92 %
N_2 = 77·2 %	N_2 = 78·67 %
CO_2/O_2 = 4·5 %	CO_2/O_2 = 1·65 %

Exp. 7. Repetition of the preceding. Duration 5 hrs. 25 cc. of each liquid.

Flask No. 1	Flask No. 2
CO_2 = 2·8 %	CO_2 = 1·56 %
O_2 = 19·29 %	O_2 = 20·04 %
N_2 = 77·91 %	N_2 = 78·4 %
CO_2/O_2 = 2·86 %	CO_2/O_2 = 2·21 %

Exp. 8. Two flasks. In each 5 g. of hefanol and 25 cc. 2 % potassium lactate. In addition, in the first 25 cc. 0·5 % methylene blue, and in the second 25 cc. water. In the thermostat at 34°. Duration of the experiment 3·5 hrs.

Flask No. 1	Flask No. 2
CO_2 = 7·83 %	CO_2 = 3·84 %
O_2 = 18·01 %	O_2 = 19·53 %
N_2 = 74·16 %	N_2 = 76·63 %
CO_2/O_2 = 4·90 %	CO_2/O_2 = 5·48 %

Notwithstanding the very thin layer of liquid, in the last three experiments the aeration was very incomplete, and high coefficients were produced. In the experiments succeeding the flasks used in the experiments 1–3 were taken. During the experiment they were shaken on a shaking apparatus.

Exp. 9. Three flasks. Duration of experiment 3 hrs.

	Flask No. 1	Flask No. 2	Flask No. 3
Hefanol	5 g.	5 g.	5 g.
Methylene blue 0·5 % ...	25 cc.	25 cc.	—
Potassium lactate 2 % ...	25 cc.	—	25 cc.
Water	—	25 cc.	25 cc.

Analysis of gases:

Flask No. 1	Flask No. 2	Flask No. 3
CO_2 = 7·74 %	CO_2 = 3·72 %	CO_2 = 3·84 %
O_2 = 15·31 %	O_2 = 19·50 %	O_2 = 19·04 %
N_2 = 76·95 %	N_2 = 76·78 %	N_2 = 77·12 %
CO_2/O_2 = 1·54 %	CO_2/O_2 = 4·83 %	CO_2/O_2 = 2·90 %

Allowing for the control experiment (No. 2) we have as the final ratio

$$\frac{7{\cdot}74-3{\cdot}72}{5{\cdot}00-0{\cdot}77}=0{\cdot}95.$$

Exp. 10. Three flasks. Duration of experiment 3·5 hrs.

	Flask No. 1	Flask No. 2	Flask No. 3
Hefanol	7 g.	7 g.	7 g.
Methylene blue 0·5 % ...	25 cc.	25 cc.	—
Potassium lactate 2 % ...	25 cc.	—	25 cc.
Water	—	25 cc.	25 cc.

Analysis of gases:

Flask No. 1	Flask No. 2	Flask No. 3
CO_2 = 9·84 %	CO_2 = 4·63 %	CO_2 = 5·77 %
O_2 = 13·45 %	O_2 = 18·44 %	O_2 = 18·91 %
N_2 = 76·71 %	N_2 = 76·93 %	N_2 = 75·32 %
CO_2/O_2 = 1·44 %	CO_2/O_2 = 2·47 %	CO_2/O_2 = 5·94 %

Allowing for the control as above we have:

$$\frac{CO_2}{O_2}=1{\cdot}06.$$

Exp. 11. Three flasks. Duration of the experiment 5 hrs.

	Flask No. 1	Flask No. 2	Flask No. 3
Hefanol	7 g.	7 g.	7 g.
Methylene blue 0·5 % ...	25 cc.	25 cc.	—
Potassium lactate 2 % ...	25 cc.	—	25 cc.
Water	—	25 cc.	25 cc.

Analysis of gases:

	Flask No. 1	Flask No. 2	Flask No. 3
$CO_2 =$	12·96 %	4·92 %	6·09 %
$O_2 =$	11·24 %	18·07 %	18·40 %
$N_2 =$	75·80 %	77·01 %	75·51 %
$CO_2/O_2 =$	1·48 %	2·18 %	3·98 %

Allowing for the control, we have:

$$\frac{CO_2}{O_2} = 0{\cdot}80.$$

The results of the last three experiments show that with good aeration the ratio CO_2/O_2 during the decomposition of potassium lactate by yeast in the presence of methylene blue is only slightly less than one.

Just as in respiration, according to the theory of Palladin, the oxygen is absorbed in order to remove the hydrogen; in the last three experiments the oxygen absorbed oxidised the hydrogen which was removed from the lactic acid by means of methylene blue. Thus, the methylene blue has taken the place of the respiratory pigment.

III. THE DECOMPOSITION OF LACTIC ACID IN THE PRESENCE OF PYRUVIC ACID.

The problem for our further experiments was to find the conditions in which the lactic acid would produce, instead of acetaldehyde, alcohol. The question then arose, what was the substance that in natural conditions substituted methylene blue during alcoholic fermentation in the process of decomposition of lactic acid? Of course, only acetaldehyde can be this substance. From our point of view the scheme of decomposition of lactic acid by yeast with the production of alcohol is represented in the following manner:

(1) $CH_3 . CH(OH) . COOH + CH_3 . CHO = CH_3 . CO . COOH + CH_3 . CH_2 . OH,$

(2) $CH_3 . CO . COOH = CO_2 + CH_3 . CHO.$

The acetaldehyde produced oxidises new quantities of lactic acid, and so on. Consequently, in the presence of an insignificant quantity of acetaldehyde it is possible to convert an indefinitely large quantity of lactic acid into alcohol. In order to test the correctness of the view expressed, we took, instead of acetaldehyde, pyruvic acid, which when decomposed by carboxylase, produces acetaldehyde:

$$(1)\quad CH_3.CH(OH).COOH + CH_3.CO.COOH = CH_3.CH(OH).COOH + CH_3.CHO + CO_2,$$

$$(2)\quad CH_3.CH(OH).COOH + CH_3.CHO + CO_2 = CH_3.CO.COOH + CH_3.CH_2.OH + CO_2.$$

We had two reasons for taking pyruvic acid. Firstly, because it produces acetaldehyde *in statu nascendi*; secondly because Oppenheimer [1914] demonstrated that pyruvic acid evokes the decomposition of lactic acid by yeast juice.

Exp. 12. Nine flasks with 5 g. of zymin in each. In addition, in three flasks 25 cc. 0·2 % pyruvic acid neutralised by caustic potash, and 25 cc. of water; in three flasks 25 cc. 2 % lactic acid neutralised by caustic potash, and 25 cc. of water; in the remaining three flasks 25 cc. 2 % lactic acid, and 25 cc. 0·2 % pyruvic acid neutralised by caustic potash. The following quantities of carbon dioxide in mg. were evolved:

	18 hours	24 hours	60 hours
1. Pyruvic acid	116	174	248
2. Lactic acid	117	164	219
3. Lactic and pyruvic acids	147	223	325

The average quantities of carbon dioxide evolved in one hour were as follows:

Duration of the experiment. Hours	Pyruvic acid		Lactic acid		Lactic and pyruvic acids	
	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.
18	116	6·5	117	6·5	147	8·2
6	58	9·5	47	7·8	76	12·6
36	74	2·0	55	1·5	102	2·8
60	248	—	219	—	325	—

Thus, the addition of a small quantity of pyruvic acid increased the evolution of carbon dioxide at the expense of lactic acid in the following manner:

18 hours. 147 − 117 = 30 (+ 25·6 %)
24 hours. 223 − 164 = 59 (+ 36·0 %)
60 hours. 325 − 219 = 106 (+ 48·4 %)

Taking into account, that from the decomposition of the pyruvic acid only 25 mg. of carbon dioxide could be evolved, it follows that the remaining 81 mg. (106 − 25 = 81) of carbon dioxide were evolved at the expense of the lactic acid.

Therefore, it is to be concluded that *pyruvic acid evokes the decomposition of lactic acid by killed yeast, though in a weaker degree than methylene blue.*

This surplus of carbon dioxide cannot be attributed to the stimulation of auto-fermentation by the pyruvic acid, as, in comparison with the quantity of carbon dioxide evolved in presence of lactic acid, in the experiments in which auto-fermentation proceeded in the presence of pyruvic acid only a slight surplus was produced, hardly exceeding the 25 mg. produced from the decomposition of pyruvic acid:

18 hours. 116 − 117 = 0
24 hours. 174 − 164 = 10
60 hours. 248 − 219 = 29

It must also be noted that the pyruvic acid stimulated the decomposition of lactic acid at first in a weak degree (25·6 %), but at the end of the experiment this stimulation gradually increased (48·4 %).

Exp. 13. Three flasks with 5 g. zymin in each. In addition, in the first flask 25 cc. 0·2 % pyruvic acid neutralised by caustic potash and 25 cc. of water, in the second flask 25 cc. 2 % lactic acid neutralised by caustic potash and 25 cc. of water, in the third flask 25 cc. of 2 % lactic acid and 25 cc. 0·2 % pyruvic acid neutralised by caustic potash. During fermentation the flasks were firmly closed with india-rubber stoppers. After 25 hours the quantity of acetaldehyde was estimated:

1.	Pyruvic acid...	13·5 mg.
2.	Lactic acid	0 „
3.	Lactic and pyruvic acids ...	11·5 „

In the third portion, according to the quantity of carbon dioxide evolved in the first experiment, it was to be expected that 59 mg. of acetaldehyde would be produced. But in reality only 11·5 mg. were produced.

It is to be concluded, therefore, that *the decomposition of lactic acid by killed yeast in the presence of pyruvic acid takes place without acetaldehyde being produced, i.e. in a different manner from that which takes place in the presence of methylene blue.*

Exp. 14. Two flasks with 5 g. zymin in each. In addition, in the first flask 25 cc. 2 % lactic acid neutralised by caustic potash and 25 cc. of water; in the second flask 25 cc. 2 % lactic acid and 25 cc. 0·2 % pyruvic acid neutralised by caustic potash. The experiment was conducted for 24 hours. In order to liberate the combined carbon dioxide, at the end of the experiment 10 cc. 10 % sulphuric acid were added to each of the two flasks. The quantity of alcohol was then estimated by the method of Nicloux.

		Carbon dioxide	Alcohol
1.	Lactic and pyruvic acids	205	167
2.	Lactic acid	138	143
		67	24

$$\frac{C_2H_6O}{CO_2}=\frac{24}{67}=0{\cdot}35.$$

Exp. 15. Three flasks with 7 g. zymin in each. In addition, in the first flask 25 cc. 0·2 % pyruvic acid neutralised by caustic potash and 25 cc. of water, in the second flask 25 cc. 2 % lactic acid neutralised by caustic potash and 25 cc. of water, in the third flask 25 cc. 2 % lactic acid and 25 cc. 0·2 % pyruvic acid neutralised by caustic potash. The experiment was conducted for 62 hours. The estimations of carbon dioxide and alcohol were made as in the preceding experiment.

		Carbon dioxide	Alcohol
1.	Pyruvic acid	490	476
2.	Lactic acid	447	445
3.	Lactic and pyruvic acids	695	514

Therefore, the third portion gave the following surplus of carbon dioxide and alcohol, as compared with the second:

Carbon dioxide 695 – 447 = 248 mg.
Alcohol ... 514 – 445 = 69 „

If we suppose that in the third portion the pyruvic acid not only evoked the decomposition of lactic acid, but also stimulated auto-fermentation, we must subtract from the figures of the third portion those of the first portion, not of the second:

Carbon dioxide 695 – 490 = 205 mg.
Alcohol ... 514 – 476 = 38 „

Therefore, even if we allow for the stimulation of the process of auto-fermentation by pyruvic acid in the presence of lactic acid, we must conclude that *pyruvic acid evokes the decomposition of lactic acid by killed yeast with the production of alcohol and carbon dioxide.* But a sharp deviation from the normal alcoholic fermentation is observed, for to a great quantity of carbon dioxide evolved, only a small quantity of alcohol is produced:

$$(1)\quad \frac{C_2H_6O}{CO_2}=\frac{69}{248}=0{\cdot}27,$$

$$(2)\quad \frac{C_2H_6O}{CO_2}=\frac{38}{205}=0{\cdot}18.$$

What has caused such a great predominance of carbon dioxide over alcohol? It is well known from organic chemistry that reactions very rarely correspond to the theoretical equations: usually the main reaction is accompanied by a greater or smaller number of accessory reactions. Moreover, all the chemical reactions studied by us in killed organisms differ more or less widely from the same reactions which take place in living organisms, in that they are accompanied by different accessory reactions, absent from living organisms.

In all probability, in the case investigated by us, the accessory reactions have also partly obscured, and partly altered, the main reaction of decomposition of lactic acid into alcohol and carbon dioxide. It is possible, that one of these accessory reactions was the reaction of Cannizzaro, very common in killed plants and animals. It is unknown whether it takes place in living organisms. This reaction alone could have reduced the production of alcohol by half. It may be also that part of the lactic acid was decomposed with the evolution of carbon dioxide, but without the formation of the aldehyde. Lastly, it is possible that the surplus of carbon dioxide was produced by further oxidation of the acetaldehyde. Further investigations are needed to solve these questions.

A surplus of carbon dioxide over alcohol is a usual phenomenon during anaerobic respiration of seminiferous plants. It is to be hoped that some of these cases will be explained after further studies on the decomposition of lactic acid by yeast in the presence of pyruvic acid.

The alcohol which was found in portions with lactic acid alone, was, of course, not produced from the latter, but appeared as the result of auto-fermentation, as the control experiment proved that the addition of neutralised lactic acid to zymin scarcely increases the quantity of carbon dioxide evolved and alcohol produced. Thus, 7 g. of zymin after 62 hours of auto-fermentation evolved 400 mg. of carbon dioxide and produced 417 mg. of alcohol.

Just as the negative results of Buchner's experiments do not prove that lactic acid cannot be the intermediate product of alcoholic fermentation; our own positive results—on account of the discovery of the wide capacity of killed yeast for decomposing the most dissimilar compounds, which are known to have no relation whatever to alcoholic fermentation[1]—do not yet conclusively prove that lactic acid is the intermediate product of alcoholic fermentation. It remains to stop the alcoholic fermentation in the stage of lactic acid, and this is what we are now engaged upon. At any rate, our experiments give, firstly, a scheme which shows in which direction the work must be conducted, and, secondly, prove that *alcohol does not present the result of decomposition, but the product of reduction of acetaldehyde by removing the hydrogen from one of the intermediate products of decomposition of glucose.*

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[1] For example, it is impossible, on account of the formation of aniline from nitrobenzene by killed yeast, to say that these substances are the normal products of metabolism in yeast.

The investigations on the significance of hydrogen during alcoholic fermentation and respiration have made a complete revolution in our ideas on these processes. They have proved that the reactions of reduction are of main importance. Firstly, on them depend the anaerobic oxidations. Secondly they have made it clear that, *during the formation of the intermediate products, in the molecule of glucose the hydrogen is displaced, and not the oxygen,* as Hoppe-Seyler supposed [1876]. Thirdly, the chemical processes of alcoholic fermentation and respiration cannot be explained by reactions of decomposition alone, as the hydrogen is displaced not only within the confines of one molecule, but also passes from one molecule into another. This means that during alcoholic fermentation and respiration the molecules act on each other reciprocally. Grey's experiments [1913] on the anaerobic fermentation of glucose by normal *Bacillus coli communis* (Escherich) and a strain derived from the normal organism by selection with sodium chloroacetate also speak in favour of the theory that lactic acid presents an intermediate product in alcoholic fermentation. The normal bacteria produce besides lactic acid,—acetaldehyde, alcohol and carbon dioxide. But artificially selected bacteria decompose glucose only as far as lactic acid, without producing either acetaldehyde or carbon dioxide, and producing very little alcohol.

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XX. NITROGEN DISTRIBUTION IN THE TISSUES AND SOME OF THE FACTORS WHICH INFLUENCE IT.

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PREVIOUS WORK.

In the development as well as in the foundation of our knowledge the importance of technique can hardly be overestimated; a new method has been the sesame to many a hitherto closed door and has opened new fields for research. One need only refer to the discovery and elaboration of the methods for the determination and isolation of the individual amino acids by Fischer, Abderhalden and others, which threw a flood of light upon the structure of the protein molecule and revealed the astounding fact of its complete cleavage in the alimentary canal by the various processes of digestion. Linked to this was the necessary corollary that the animal organism was capable of resynthesising protein out of its cleavage products. There was everything in favour of the further assumption that it would be able to maintain itself and build up the required tissue protein if the protein of the food were entirely replaced by its cleavage products or by a mixture of its component amino acids. Abderhalden [1912, 1] in one of his papers, dealing with the synthetic capacities of the tissues, gives an account of the various steps by which he was able to feed a dog on completely cleaved protein and finally on an artificial mixture of amino acids for periods of eight and twelve days in two successive experiments; in both cases nitrogen equilibrium was established and there was a very slight increase in weight. An examination of these experiments shows that the trypsin digests from dog, ox and horse protein were more satisfactory substitutes for protein than the amino acid mixture, for the result of their administration was frequently a marked retention of nitrogen accompanied by a gain in weight. A gliadin digest was markedly inferior to the animal digests doubtless owing to the relative amounts of the constituent amino acids differing widely from those of the tissues. One very interesting experiment [Abderhalden, 1913] demonstrated in a striking way that protein cleavage products were not only capable of replacing tissue waste but that they sufficed also for the new formation of tissue; a dog which had fasted for 26 days was shaved and then fed on a diet which contained all its nitrogen in the form of completely digested flesh. Not only did the dog put on 10 kilos. weight but it grew a new coat showing unmistakably that a synthesis of protein from the amino acid mixture had taken place. In some experiments which were carried out by Buglia [1912, 1] the meat in the dietary of young growing dogs was replaced by meat digests without affecting the retention of nitrogen or the gain in weight, provided that the carbohydrate in the food was given in a readily absorbable form. Frank and Schittenhelm [1910, 1911], in a series of experiments which will be referred to later, found that the digests of various forms of protein were almost as efficient as the proteins themselves, and in the case of one of the best utilised proteins, egg albumin, the digest was actually as efficient as the undigested albumin.

The attention of physiologists having been directed to the synthetic capacities of the organism, it was only a step further to inquire whether the amino acids themselves could be built up out of simpler forms of nitrogen. The work of Knoop and Kertess [1911] and of Embden and Schmitz [1912] acted as a fresh stimulus in this direction. Knoop and Kertess had found that the injection of an α-keto-acid or of an α-hydroxy-acid was followed by the appearance of the corresponding amino acid in the urine showing that nitrogen had been added on in the tissues. Embden and Schmitz perfused the isolated glycogen-free liver with the ammonium salt of an α-keto-acid and were able to recover from the perfusing fluid the corresponding amino acid, and they further obtained alanine after perfusing ammonium chloride through a glycogen-rich liver.

It is now generally accepted that glycocoll can be synthesised in the rabbit [Abderhalden and Hirsch, 1912, 1] but the results of replacing the protein of the food by an ammonium salt or urea have not been as satisfactory as might reasonably have been expected if the tissues can readily synthesise the amino acids necessary for the further construction of protein. The main workers in this field have been Grafe [1912, 1, 2, 1913, 1, 2, 3, 1914] and Abderhalden [1912, 1, 2, 1913]. Grafe seems to have started out with the definite expectation of finding that some, at any rate, of the food protein could be replaced by nitrogen in the form of ammonia or urea, provided that an abundant carbohydrate fat diet was supplied, but the large number of experiments performed, coupled with the statement that some of them were not as clear as he wished, indicates that he had difficulty in convincing himself that an unequivocal synthesis had occurred. Grafe [Grafe and Schläpfer, 1912] planned his experiments with great care and the conditions under which they were carried out were practically always the same, viz., the animal was prepared by a previous fast of some days, following which there was a pre-period, in which the diet was nitrogen free and consisted of abundant carbohydrate and fat to the amount of 150 or more calories per kilo. body weight. The effect of this was of course to reduce the nitrogen metabolism to a minimum. The mean of the last three days of the pre-period and of the first three days of a similar after-period served as a measure of the endogenous metabolism, and was used to determine the nitrogen retention in the principal period during which nitrogen was added to the carbohydrate fat diet in the form of an ammonium salt or [Grafe and Turban, 1913] of urea. By this method Grafe was able to secure a well-marked reduction of a negative nitrogen balance and an occasional positive balance on particular days in the principal period. He claimed that very considerable amounts of nitrogen were retained, *e.g.* in one experiment [1912, 2] on a pig, 14 g. of ammonium citrate nitrogen were retained in 16 days. Young growing pigs proved more suitable for these experiments than dogs because they took the food readily and put on weight with astounding rapidity. It is noteworthy as showing how long life can be maintained in a state of complete nitrogen starvation, that two young pigs [1913, 3] of the same litter, one of which was fed on carbohydrate and fat alone, while urea and ammonium citrate were added to this diet in the case of the second, both died on the 67th day of the experiment although the animal which received no nitrogen began to fail much earlier than the other.

Grafe's [1913, 1] most favourable results were obtained in his later experiments by a modification of the procedure; after reducing the nitrogen metabolism to a minimum in the usual way, in a further pre-period he gave a small amount of protein equal to from 0·5 to 0·7 of the maintenance minimum, which was continued in the principal period during the administration of urea alone or of ammonium citrate and urea. The nitrogen balance remained negative in the pre-period in spite of the small quantity of protein but it became definitely positive in the principal period, *e.g.* pig 8 had an average daily balance of −0·64 g. in the pre-period which became + 0·812 g. in the principal period. Grafe points out the importance of making the difference between the nitrogen fed in the form of ammonium salt and urea on the one hand and the protein on the other as great in amount as possible, stating that it should be 3·5 times the value of the maintenance minimum and he criticises the small amount (1·3 times the maintenance minimum) used by Abderhalden and Lampe [1913, 1]. It is interesting to note that Grafe [1913, 2] found a negative daily balance of − 0·18 g. in a pig fed for 40 days with 4·7 g. of urea nitrogen daily as the sole source of nitrogen. In a later paper [1913, 3] he ran parallel experiments with pigs of the same litter; pig 15 was given rather less than the maintenance minimum of protein during the principal period, pig 16, on the other hand, received in addition urea and ammonium citrate; both pigs gained in weight but the balance remained negative in pig 15 and became positive in pig 16. In a second experiment pig 17 got rather more than the maintenance minimum of protein in the principal period, while pig 18 received no protein but only ammonium citrate and urea; both pigs behaved in exactly the same way putting on weight and exhibiting a positive nitrogen balance.

Grafe [1914] found that a certain amount of nitrogen retention also occurred when an inorganic salt, ammonium chloride, was substituted for the organic salt, provided that it was given in small or medium doses; in larger doses it exerted an injurious influence. An endeavour to satisfy the nitrogen requirements of the animal by giving it in the form of nitrate [Grafe and Wintz, 1913] led to most contradictory results partly owing to the injurious effect of the nitrate itself. Abderhalden and Hirsch [1913] found that sodium nitrate was quantitatively excreted in the urine in the case of four dogs; in spite of this the nitrogen balance, exclusive of the nitrate, was affected favourably in two and unfavourably in the other two dogs.

Grafe [1913, 3] mentions three possibilities with regard to the nitrogen retained; ammonia may be retained as such, or the ammonia may be anchored, *i.e.* it enters into some form of combination not protein, and lastly ammonia may spare body protein in some way, *i.e.* ammonia is retained in some protein-like form. He decides in favour of the nitrogen being retained in a protein-like form.

Abderhalden and his co-workers Hirsch and Lampe carried out their experiments on the same general principles as Grafe, but the experiments differed in various details and were not planned with an equal uniformity. It is often difficult and tedious to follow the tables owing to the frequent absence of the average daily balance calculated for the various periods under consideration. The first results were published in 1912, immediately after the appearance of the paper by Grafe and Schläpfer; they dealt with the effects of superimposing ammonium carbonate or ammonium citrate on an abundant carbohydrate fat diet. Although the nitrogen balance remained negative some of the added nitrogen was undoubtedly retained. Loss of weight continued in spite of the feeding with ammonia, but in two instances when a blood or flesh digest was added to the nitrogen-free diet the weight rose and the balance became definitely positive. Shortly after this there appeared an account [Abderhalden and Hirsch, 1912, 2] of two experiments which lasted 103 and 111 days respectively in which ammonium salts were added to a nitrogen-free diet. The difficulty of the continued administration of ammonium salts due to vomiting and diarrhoea necessitated the frequent interruption of the ammonia periods and the intercalation of fast days. Sometimes the condition of the animal was such that feeding with meat powder or flesh digest for some days had to be resorted to, a procedure which was invariably followed by immediate recovery. A retention of some of the ingested ammonia undoubtedly occurred, although the authors urged caution in the interpretation of the results on account of the variations which were present in the daily nitrogen balance. They drew attention to the fact that a temporary gain in weight might be exhibited under certain conditions by an animal fed on a nitrogen-free diet. With regard to the utilisation of urea [Abderhalden and Hirsch, 1912, 4] any retention of nitrogen in the dog was followed by a washing out. In an isolated experiment [Abderhalden and Lampe, 1913, 2] on a pig there was not even a temporary holding back of nitrogen for the negative daily balance was practically the same as that of the succeeding fast days; it may be mentioned however that the nitrogen metabolism had not been reduced to a minimum by a pre-period of a nitrogen-free diet. A pig [Abderhalden and Lampe, 1913, 1] fed for 28 days on ammonium acetate, on the other hand, showed a definite protein sparing action of the ammonia although the balance remained negative.

Abderhalden and Lampe [1912, 2] criticised Grafe's findings and methods and complained that they were unable to obtain such favourable results. In their own experiments they stated that a positive nitrogen balance or nitrogen equilibrium was only transitory and was followed by an increased output. They admitted that ammonium salts exercised a protein sparing action but considered that the facts were best explained by the retention of the nitrogen in a non-protein form. They doubted [1912, 1] the existence of any marked synthetic capacity on the part of the tissues on the ground that experiments on dogs, in which the nitrogen was supplied in the form of gelatin and ammonia, failed to show a positive nitrogen balance although there was undoubted retention of nitrogen. They maintained that if the cells could synthesise amino acids a much better result should be gained if the task imposed on them were merely that of synthesising the few amino acids which were lacking in the gelatin, than if they were required to synthesise the whole series present in the protein molecule.

Following on this paper came another [Abderhalden and Hirsch, 1912, 3] in which the effect of gelatin plus ammonia was compared with that of erepton (flesh digest) on a three months old dog. A positive nitrogen balance and a gain in weight always occurred when erepton was fed, whereas the weight fell and the balance became negative when it was replaced by gelatin with ammonia. Four young dogs were then fed for periods of seven days on (1) gelatin and ammonium acetate, (2) erepton, (3) an artificial mixture of amino acids, (4) erepton and (5) ammonium acetate successively. The weight of the animals and their general condition only was noted; the weight rose in the two erepton periods, remained stationary during the feeding with amino acids and fell when ammonium acetate was given either with or without gelatin. The animals were cheerful and well during the erepton periods but they slept all day and seemed definitely unwell during the administration of ammonia.

It is quite apparent from a consideration of the foregoing experiments that even if the organism can synthesise amino acids from ammonia and the products of carbohydrate and fat metabolism, it does not readily do so, for flesh digests are undoubtedly more satisfactory than ammonium salts or urea as sources of nitrogen supply.

In connection with the utilisation of ammonium salts Underhill and Goldschmidt [1913, 1] carried out some useful work and pointed out the advisability of estimating the ammonia as well as the total nitrogen. The type of experiment differed from those just described, for the dogs were fed on an adequate mixed diet and the particular ammonium salt was administered on one day only in three separate portions in gelatin capsules. A radical difference was found in the behaviour of the organic and the inorganic salts; none of the organic salt was excreted as ammonia and it was apparently quantitatively converted into urea. About 20 to 25 per cent. of the inorganic salt, on the other hand, was excreted as urea while the remainder appeared as ammonia in the urine. There was, moreover, an increase in the nitrogen output and a lag in the elimination of the inorganic salt for the ammonia in the urine was increased for 48 hours after its administration.

A similar experiment [1913, 2] carried out on a fasting dog which had been freed from glycogen gave results in complete accord with the above. They [1913, 3] finally tested the utilisation of ammonium salts with a non-nitrogenous diet using Grafe's method. Their results were in keeping with those of previous workers in the case of the organic salts, which caused a retention of nitrogen and did not increase the urinary nitrogen, but contrary to the conclusion of Grafe and Schläpfer they found that ammonium chloride was incapable of acting as a source of nitrogen supply for the nutritional needs of the body, and not only was there an increased excretion of nitrogen, but for some days following the administration of the salt the ammonia in the urine was increased.

The authors criticise Grafe's technique particularly with regard to the absence of catheterisation, but in view of the duration and the number of Grafe's experiments and the care and consistency which characterise them his main results cannot be called in question.

Taylor and Ringer [1913] pointed out that the presence of carbohydrates was not an obligatory factor in the retention of nitrogen from ammonia. The administration of a single dose of ammonium carbonate to a dog, which had previously fasted three days, was accompanied by a retention of a considerable portion of it, the result being confirmed by two repetitions, while the retention in the case of two phloridzinised dogs was even greater. They claim that two administrations of urea were followed by a quantitative elimination of the amide, but in view of the variation in the daily nitrogen balance such a result requires confirmation. They suggest that ammonia nitrogen is retained because of a reversed reaction that leads to its combination with the α-ketonic and α-hydroxy-acids to form amino acids, which may be used in the synthesis or sparing of body protein. It cannot be said that these experiments have disproved Grafe's contention that the administration of abundant carbohydrate is the surest way of demonstrating a retention of ammonia nitrogen in the normal dog and pig.

Henriques and Anderson [1914, 1] attacked the problem of the utilisation of ammonium salts and urea in another way which excluded the action of the intestinal wall and of the intestinal bacteria. Using their method of permanent intravenous injection in the goat they reproduced Grafe's experiments, *i.e.* there was a daily injection of two litres of fluid containing glucose and salts in a pre- and after-period, and intercalated between the two was a period in which urea or an ammonium salt was added to the injection. Although there was a diminution of the negative balance, a lasting retention of nitrogen was absent and the evidence indicated a retention followed by a washing out. When a meat digest was added to the fluid a positive balance was at once obtained emphasising the superiority of digests over ammonium salts to which attention has already been drawn.

It must not be forgotten that a great deal of work on the utilisation of ammonium salts and amides has been carried out on the herbivora and in their case no doubt exists as to the effect on the nitrogen balance. Among the recent workers Kellner, Eisenkolbe, Flebbe and Neumann [1910] conclude from their experiments that asparagine and ammonium acetate can replace the protein necessary for maintenance but that they cannot be utilised for protein formation even in starving animals.

Morgan, Beger and Westhausser [1910] state that ammonium acetate can be utilised in no inconsiderable way for the maintenance of life and even for milk formation if it be added to a diet containing sufficient starch but very deficient in protein. In a later paper [1911] they found that the nitrogen contained in ammonium acetate and asparagine in a diet poor in protein but with sufficient nitrogen-free food was utilised to the extent of 32 per cent. not only for the maintenance of life but also for the production of milk, flesh and wool. Probably the intestinal bacteria play an important part in the utilisation of these simple nitrogenous bodies in the herbivora for the value of these as food stuffs is admitted.

The complexity of the problems of protein metabolism is indicated by the apparent difference which exists between the processes of tissue repair and growth; various workers have shown that food which suffices for repair is not necessarily capable of producing growth. McCollum [1911] reduced the metabolism of a pig to a minimum by feeding it on a scanty starch and salt diet after a preliminary fast. The creatinine nitrogen under these conditions constituted 1/5·5 of the total nitrogen and at this low level all the nitrogen was assumed to be of endogenous origin; the creatinine nitrogen × 5·5 therefore was used as a measure of endogenous metabolism in the subsequent calculations and it was possible to determine how much of this endogenous or tissue nitrogen had been replaced by the nitrogen in the substance fed. It was found that zein was utilised to the extent of 80 per cent. on an average for repair, but even when given in excess there was no evidence of the formation of body tissue. The average utilisation of gelatin for repair was 50–60 per cent., whereas when caseinogen was the only protein fed there were increases of body protein to the extent of 20–25 per cent.

Osborne and Mendel [1912, 2] carried out experiments on the feeding of isolated proteins to rats. Gliadin, which contains relatively very little glycocoll, arginine, histidine or lysine and extremely large quantities of glutamic acid, proline and ammonia, was unable to support normal growth in young growing rats who were dependent on it for their sole nitrogen supply. The same diet however sufficed for the maintenance of the adult animals, which not only paired but also produced healthy young, which were suckled by the mother, so that in this case there must have been a synthesis in the maternal organism of the amino acids which were lacking in the gliadin. Of four young rats suckled by a gliadin-fed mother, the three which were taken away from her at the end of 30 days and were then fed on caseinogen, edestin and milk respectively, showed normal growth, while the fourth, which was left in the cage with the mother and had access only to the gliadin mixture, showed no growth. Hordein also was incapable of maintaining normal growth in contradistinction to caseinogen, edestin and glutenin. As Osborne and Mendel point out maintenance experiments alone are not sufficient to solve the problem of the biochemical value of dietaries. An account appeared elsewhere [1912, 1] of an elaborate investigation which led to an extension of the list of proteins which were capable of supporting normal growth. The soy bean was the only member of the Leguminosae containing a protein which fulfilled these conditions. Hordein and zein resembled gliadin in their inability to do so. With regard to the quantity of protein it was found that normal growth occurred if 18 per cent. of protein (or 14 to 15 per cent. calories) was present in the diet, but there was no increased growth if this quantity was increased. Frank and Schittenhelm [1910] investigated the question of the superiority of the "arteignes" protein of Michaud for tissue repair over allied forms of protein. They reduced the nitrogen metabolism of dogs to a very low level by the use of a nitrogen-free, carbohydrate-rich diet following a fast and then tested the relative values of dog, horse, pig, fish, goose and pigeon flesh. In two out of the five dogs, dog protein was the best utilised but in the other three dogs one of the other forms of protein held the first place. They concluded that "arteignes" protein had no definite superiority over other nearly allied proteins provided they could be readily broken down and that they yielded much the same mixture of amino acids. Continuing this line of work [1911] they succeeded in maintaining without loss of weight two dogs for 6·5 and 4·5 months respectively on a diet in which the nitrogen minimum was given in the form of different types of animal protein or their digests. They found no marked difference in the utilisation of the different types of meat and the digests were almost as efficient as the undigested proteins. One dog with an intake of 1·2 g. protein was practically continuously in nitrogen equilibrium or else showed a positive balance, in the second dog a positive balance was not reached nearly so soon.

The method of introducing food stuffs intravenously has been practised by a number of workers and has had for its object the exclusion of the action of the intestinal wall. Buglia [1912, 2] injected meat digests intravenously in dogs and cited as evidence of their utilisation that only varying proportions were excreted in amino acid form in the urine. The experiments lack uniformity and are few in number but such a utilisation is in harmony with the results of the work of Folin and Denis [1912] and van Slyke and Meyer [1912, 1913, 1, 2, 3] who showed that the products of digestion were carried to the tissues as such without undergoing any preliminary change in the intestinal wall.

Henriques and Anderson [1913] elaborated the method of intravenous injection and were able to keep a goat alive for 20 days by the daily introduction of protein digests into the blood stream; the nitrogen balance under these conditions was frequently positive and a daily retention of 1 or 2 g. of nitrogen was quite common. Their use of this method in the investigation of the utilisation of urea and ammonia has already been referred to.

The same observers [1914, 2] used their method of permanent intravenous injection in goats after total extirpation of the bowel. They managed to keep such animals alive for 2½ days. During this time the injection of trypsin + erepsin digests caused a considerable deposit of nitrogen, for example in goat 1 positive balances of 2·2 g. and 4·18 g. were obtained on the first and second day respectively.

Oehme [1914] injected meat digests intravenously on a single day in the case of two fed and two fasting dogs respectively, the diet of the former consisting of carbohydrate and fat. The amount of amino acid excreted unchanged in the urine was much smaller in the fed than in the fasting dog but even in the latter the protein cleavage products injected had been extensively utilised, for the maximum excretion of amino acid was only 29·5 per cent. of the amount injected. In view of the capacity of the tissues to pick up amino acids, etc. from the blood stream the fact of a certain amount of the amino acid introduced not appearing within a given time in the urine certainly cannot be regarded as evidence of its utilisation. The real aim of the researches on the introduction of food stuffs intravenously was to obtain evidence that the intervention of the intestinal wall was not necessary and to disprove the hypothesis enunciated by Abderhalden of a synthesis of the products of digestion by the cells of the intestine.

When we come to the field of tissue analysis mention must first be made of the work of Grund [1910] and Diesselhorst [1911]. Grund endeavoured to find evidence of protein storage in the liver of the dog as it had been claimed by Seitz that such a storage of protein took place to a very marked extent in the liver of the hen. Grund estimated the total nitrogen, the total protein nitrogen and the total phosphorus in the liver, kidney and muscle of the fed and fasting animals, and compared the two values. When the total nitrogen was calculated as a percentage of the initial body weight, there was a great diminution in all these organs but particularly in the case of the liver as the following figures show:

	Liver	Kidney	Muscle
Mean of the fed dog ...	1·059 %	0·157 %	0·185 %
Mean of the fasting dog ...	0·570 %	0·098 %	0·129 %

If the percentage of total nitrogen in the liver of the fed and fasting animals be compared, however, the increase is in the fasting animal, due presumably to a diminution of glycogen in the latter which would lead to a relative increase in the total nitrogen content of the organ. Changes in the amount of moisture are not accountable for the increase. The kidney and muscle show practically no change in the percentage of total nitrogen. Grund mentions the fact that the ratio of "rest" nitrogen (= total soluble nitrogen) to total nitrogen expressed as a percentage of the latter showed great irregularities in the individual experiments but only moderate differences between the fasting and fed animals; the average figures are:

	Liver	Kidney	Muscle
Fed animal ...	11·6 %	15·7 %	15·1 %
Fasting animal...	10·5 %	12·9 %	13·2 %

The ratio of the total phosphorus to the total nitrogen was practically constant in the organs of the fed and fasting animals. The difficulty of interpreting Grund's figures lies in the impossibility of getting a fixed basis for comparison, both the initial weight of the body and the weight of the individual organs being subject to variation from various factors. As we shall see later it is much easier to detect changes in the "rest" nitrogen than in the total nitrogen.

Diesselhorst tried to determine whether the diet influenced the chemical composition of the tissues by a comparison of the muscles of a dog fed on a scanty carbohydrate fat diet and an abundant meat diet respectively. Two dogs were fed on the meagre nitrogen-free diet for 1½ to 2 months, a hind leg was then amputated in the case of each animal and both were fed on an abundant meat diet for approximately three months. During this period they rapidly put on weight and at its termination they were killed. The main fact which emerges from the experiment is that the nitrogen : carbon ratio remains unaltered and is practically the same in the two dogs, 1 : 32 and 1 : 31·8; these figures agree closely with the ratio of 1 : 33·8 obtained by a previous worker, Stockhausen.

With the work of Folin and van Slyke we turn over a new page in the history of protein metabolism, for it has solved the problem of the form in which the products of digestion enter the blood stream and the theory of a resynthesis in the intestinal wall has been relegated to oblivion. Folin [Folin and Denis, 1912, 1] was the first to publish his results dealing with the application to the blood and tissues of his new micro-methods for the determination of total nitrogen, urea and ammonia nitrogen. In his investigation of the effects on the blood and tissues of the absorption of the products of protein digestion he selected the cat as being the most suitable animal for his experiments. After taking samples of blood and removing the gracilis muscle from one leg, he introduced into a ligatured loop of the intestine solutions of urea, glycocoll, egg albumin and a pancreatic digest respectively; samples of blood were withdrawn at varying intervals during the experiment and at its close the gracilis was removed from the other leg. The determination of the total non-protein nitrogen and the urea in the samples thus obtained showed that there had been a rapid absorption of the urea from the intestine, that it had entered into the blood stream unchanged and had actually accumulated in the muscle. In the case of the glycocoll, the increase of the non-protein nitrogen in the blood and muscle showed that it also had entered the blood stream and had travelled unchanged to the muscle, the urea content of the blood, however, was slightly increased towards the end of the experiment indicating a conversion of some of the glycocoll into this substance. In the same way there was evidence of the direct transference to the tissues of the egg albumin and protein digest. In a later paper [1912, 3] by allowing a longer interval, 2·5 to 3 hours, to elapse before the final samples of blood and muscle were taken Folin gave ample demonstration of the conversion into urea of some of the absorbed alanine, glycocoll and peptone, for, in addition to the increase of the non-protein nitrogen, there was also an increase in the urea of the blood and tissues. The absorption of creatine and creatinine on the other hand was unaccompanied by any other change than the accumulation of the non-protein nitrogen in the blood and muscle. The author points out that the liver has not brought about any specialised deaminisation of the amino acids and emphasises the fact that the urea forming process is characteristic of all the tissues.

A few experiments [1912, 1] on the difference in the non-protein and urea nitrogen content of the blood in the fasting and the fed animals provided very scanty data for drawing satisfactory conclusions as to the influence of feeding. The amount of non-protein and urea nitrogen, however, in the blood of cats fed on a nitrogen-free, carbohydrate fat diet was undoubtedly less than in the case of meat-fed cats and this result was confirmed by data in a later paper [1912, 3] where very low values were obtained after feeding cats on a rice and cream diet.

Folin had always been interested in the question of the deaminisation of the products of protein digestion in the intestinal wall and he succeeded in demonstrating in a striking way that the ammonia in the portal blood did not originate in any such process. He [Folin and Denis, 1912, 2] compared the ammonia content of the blood in the mesenteric branches of the portal vein and discovered that it was two or three times greater in the blood coming from the large intestine than in that from the small intestine, owing to the extraordinarily high ammonia content of the faeces in the large intestine, particularly after a meat diet. This coupled with the fact that the injection of a pancreatic digest, glycocoll or asparagine caused no increase in the ammonia of the portal blood was conclusive proof of the source of the portal ammonia. In connection with the question of absorption Folin [Folin and Denis, 1912, 4, 5] also showed that a certain amount of absorption both from the stomach and large intestine occurred after the introduction of solutions of amino acids, urea or peptone.

Van Slyke's work dealing with the fate of protein digestion products in the body was made possible by the elaboration of a new method [1913, 1] for the determination of amino acid nitrogen and is of the highest importance as it gives a direct method of demonstrating the presence of amino acid nitrogen—in Folin's work the presence of the amino acid was only indirectly inferred from the increase in the non-protein nitrogen. Van Slyke established the following facts in his first paper [van Slyke and Meyer, 1912], which was essentially preliminary. The blood of normal dogs, which have fasted for 20 to 24 hours, contains from 3 to 5 mg. amino acid nitrogen per 100 cc. When 12 g. of alanine were injected into the circulation of a 14 kilo. dog in 13 minutes, the added amount of amino acid nitrogen in the blood diminished very rapidly, only a relatively small portion of the alanine (1·5 g.) being excreted in the urine. It was calculated, on the assumption that the total volume of the blood was 1/20 of the body weight, that five minutes after the injection only 1·47 g. of alanine remained in the circulation. When alanine was injected into the small intestine its direct absorption was proved by the increase of the amino acid nitrogen in the blood of the mesenteric vein. The amino acid content of the blood five hours after a meal of 1 kilo. meat was practically doubled in amount. In view of these facts van Slyke is amply justified in his conclusion that the amino acids are not synthesised in the bowel wall but are offered to the tissues directly. The elaboration of these results in a later paper [van Slyke and Meyer, 1913, 1] showed that the amino acid nitrogen of the tissues was increased by an intravenous injection of amino acid. Apparently however there is a saturation point which sets the limit to absorption, as the authors say they were never able to force the figure for muscle up beyond 80 mg. per 100 g., the saturation point for liver is much higher and amounts to 125 to 150 mg. By increasing the length of the experiment [van Slyke and Meyer, 1913, 2] and comparing the amino acid content of samples of the blood and tissues taken 30 minutes after the cessation of the injection with that of samples taken several hours later, it was found that the amino acid content of the liver rapidly decreased. Although the latter might have been practically doubled in amount by the injection, the liver was able to desaturate itself completely in two or three hours, whereas the amino acid content of the muscle remained practically unaltered during this period. The disappearance of the amino acid from the blood was accompanied by an increase in the amount of urea.

A short study [van Slyke and Meyer, 1913, 3] of the effects of fasting and feeding on the amino acid content of the tissues lacks sufficient data to establish any very definite conclusions, in view of the variations present in both fasting and fed animals. The figures show that the amino acid nitrogen in the tissues of dogs fed on a protein-rich diet was not greater than the amount found in some of the fasting dogs. A nitrogen-free, abundant carbohydrate fat diet was not included in the list but it is extremely likely in view of Folin's work that under such conditions the amino acid nitrogen of the blood and tissues would have been lower than in either the fasting or the fed animal.

The following researches owe their origin to the work of Folin or of van Slyke. In their attempts to prove that the liver is not the main site of urea formation and to belittle its activity in this direction, Fiske and Sumner [1914] performed some experiments on cats, in which they completely excluded the liver from the circulation and subsequently introduced an amino acid into the blood stream. Under these conditions they produced a certain amount of evidence that urea formation still goes on. They claim that the accumulation of urea per unit mass of blood and tissues is as great in the absence of shock when the liver and other abdominal viscera are excluded from the circulation as when they are in their normal relations. It may be pointed out that the initial variation in the urea content of the blood and tissues is astounding, and one is tempted to ask whether the small increase in the values in the case of the muscle is not within the limits of experimental error.

In conjunction with Karsner, Fiske had previously [1913] carried out a few perfusion experiments on the cat's liver, ammonium carbonate or glycocoll being added to the perfusing fluid, but the experiments are of little value. The failure to find evidence of urea formation from the glycocoll can be attributed to the short duration of the experiments and to the relatively small amounts of glycocoll used. The whole amount of ammonia perfused was not converted into urea, and simply from a consideration of the figures for the total soluble nitrogen the authors regard it as doubtful if the liver has bound any ammonia as such. It may be pointed out that indirect calculations of this kind are valueless in the absence of a direct tissue analysis; the fixation of ammonia and of amino acids by the liver is an established fact.

Matthews and Nelson [1914] devised a method for determining the exact character of the metabolic changes taking place in muscle tissue. They removed practically all the viscera with the exception of the kidneys and about 20 to 25 g. of liver in decerebrate dogs, and then injected continuously into the veins a diuretic salt solution to wash all the nitrogen out of the tissues and to keep up an even flow of urine; the "zero" urine thus obtained had a very low nitrogen content and was ammonia-free. 10 g. of erepton containing only traces of ammonia and no urea were then injected in 30 minutes into the muscles. From a comparison of the urine secreted after the injection with the zero urine the authors claim to have obtained positive evidence that the amino acid mixture injected is broken down and appears in the urine largely as ammonia and urea. The data are very scanty however and the results are not concordant, *e.g.* in dog 3, Table II, the urea is actually diminished after the injection of erepton, and in dog 4 the increase is trivial.

Marshall and Davies [1914] showed that the concentration of urea in the blood and tissues was approximately the same both normally and after the injection of large quantities of urea. The following figures may be quoted:

	Normal dog	After repeated injections of ascending doses	After injection of 5 g. urea in 7 minutes
Blood	28 mg. 27 „	1065 mg.	—
Blood serum	29 „ 29 „	1034 „	493 mg.
Liver	32 „ 23 „	930 „	354 „
Muscle	25 „ 25 „	914 „	377 „

According to these workers the tissues take up with great regularity about 90 per cent. of the amount of urea injected within three minutes after the injection, whether the concentration in the body be high or low. Diffusion of the urea was practically instantaneous and was complete in all parts of the body in 15 minutes. The rate of elimination by the kidneys was very rapid and rose to 16 g. per kilo. body weight or higher per day; when the excretion of urea was prevented the entire amount was stored in the body. No evidence was found of the conversion of urea into any other substance.

McLean and Selling [1914] give some interesting data regarding the urea and non-protein nitrogen in human blood; they found that the concentration of these two forms of nitrogen was not constant in normal individuals but fluctuated from hour to hour and varied within wide limits owing to such factors as diet and the amount of fluid ingested. The figures for the urea nitrogen in their tables range from 10 to 23 mg. per 100 cc. giving a variation of ± 35 per cent. (*i.e.* the total variation is expressed as a percentage of the mean of the two extreme values), the figures for the non-protein nitrogen vary from 23 to 36 mg. per 100 cc.

Paul György and Edgard Zunz [1915] have recently studied the amino acid content of the blood of normal dogs using the van Slyke method. This value, viz. 4 to 5 mg. per 100 cc., proved remarkably constant, it was not affected by a meal of potato puree but after a meat meal (25 g. meat per kilo. body weight) the average rose to 11·9 mg. A comparison of the whole blood with the blood plasma showed that this increase was more marked in the case of the blood corpuscles than of the plasma.

Jansen [1915] as a result of perfusing the livers of 26 cats and dogs with blood of varying dilution to which, with two exceptions, additions of leucine, glycocoll, alanine, ammonium carbonate or erepton were made, came to the conclusion that, contrary to the opinion of Fiske and Karsner, the liver played an important part in the formation of urea from amino acids.

In considering the significance of the nitrogen retention which undoubtedly occurs when ammonium salts are introduced into the dietary, the rôle of ammonia in maintaining the normal neutrality of the tissues must not be forgotten. The effect of alkali on autolysis in vitro is to retard the process, and acid on the other hand has an accelerating action. Schryver [1906] obtained practically the same results in the living animal and he believed that the rôle of ammonia in retarding the activity of the proteolytic enzymes of the tissues was a very important one. That large amounts of acid can be introduced into the body by the use of certain foods with a preponderance of acid ash is evident from the work of Shermann and Gettler [1912]; these observers were able to introduce the equivalent of 21·7 cc. and 32·7 cc. N. acid per day on two occasions respectively by substituting rice for potato in a man's diet. The extent to which the acid excreted in the urine is neutralised by the ammonia present in the body is still the subject of conflicting views; in the experiment just mentioned Shermann and Gettler found that the ammonia excreted only accounted for one-quarter to one-half of the acid excreted. Janney [1911] claims that by giving large doses of alkali he was practically able to banish ammonia from the urine.

Lawrence Henderson [1911] finds that the alkalinity of the urine is always lower than that of the blood, and the amount of alkali which must be added to the urine to equalise the two represents the amount of alkali saved to the body. Under normal circumstances he states that the two factors, the physico-chemical separation of acid and ammonia secretion, are probably of the same order of magnitude and are strictly additive. Gammeltoft [1911] turned his attention to the relation of ammonia to the acid secretion of the stomach and continued the investigations of previous workers. Corresponding with the secretion of an acid juice after a meal he found a diminution in the excretion of ammonia in the urine, while following on the subsequent pouring out of an alkaline juice the ammonia excretion rose again.

In view of these facts it is evident that ammonia is constantly needed to replace the amount excreted in the urine. Now as the only source of ammonia under normal conditions is the protein of the food, it follows that whenever protein is wholly withdrawn from the dietary a shortage of ammonia in the body must occur which will become more pronounced the longer the protein-free diet is continued. Under these conditions the introduction of organic ammonium salts would inevitably lead to a retention in order to restore the normal equilibrium in the tissues. The same argument applies when a shortage of other forms of nitrogen, *e.g.* amino acid nitrogen, has been produced by withholding all nitrogen from the food; given such a condition of nitrogen hunger, the introduction of protein itself or of its cleavage products will almost certainly lead to a retention of nitrogen. In all these experiments in which the nitrogen metabolism of the animal is reduced to a very low level by the administration of nitrogen-free diet, whether it be preceded by a fast or not, this consideration must be borne in mind otherwise the results will receive too favourable an interpretation. Attention has been called to the influence of the previous state of nutrition on the course of metabolic experiments by Pfeiffer and Kriske [1911] in their investigation of the effect on sheep of a protein-rich and a protein-poor diet respectively. It was found that the retention of nitrogen continued longer and was greater in amount in the case of those animals which were in the poorest state of nutrition at the outset of the experiment. In the course of this research, which combined tissue analysis with metabolic work, the authors made an important observation which has a direct bearing on the interpretation of metabolic experiments in general; they stated that the nitrogen retention recorded in the metabolism experiments was usually greater in amount than could be accounted for by the subsequent analysis of the tissue on the slaughter of the animal.

From the experiments of Folin and van Slyke it would seem that the tissues have no selective action with regard to the various forms of nitrogen carried to them by the blood stream, but that they take up amounts of amino acid, urea and ammonia which depend chiefly on the concentration of these bodies in the blood, the higher the concentration the greater the probability will be of finding a fixation on the part of the tissues. In the experiments already mentioned in which ammonium salts were fed, the concentration of ammonia in the blood would certainly be increased above the normal immediately after its absorption and such a condition in itself would favour a retention of nitrogen. A consideration of the facts which have been enumerated should be sufficient to show what caution is needed in the interpretation of the result of metabolic experiments particularly with regard to attributing a retention of nitrogen to protein synthesis.

Turning to the tissues themselves, as we have seen under normal circumstances, a definite percentage of ammonia, amide and amino acid nitrogen is present which can readily be extracted, and although there is a considerable degree of variation in the actual and relative amount of these various substances the tissues never get entirely depleted. It is to be supposed that the amino acids present have been mainly derived from the proteins of the food and have been absorbed as such, while a small proportion has probably come from the catabolic processes of the cells themselves. Some of these amino acids must be destined for constructive and others for degradation purposes, only a very small proportion being normally excreted unchanged in the urine. Possibly the percentage of amino acid normally present in the tissues represents the point of equilibrium between the anabolic and catabolic processes in the cell. The amide has presumably originated in the tissues themselves mainly from the amino acid of the food but in a lesser degree as a degradation product of cell metabolism. The percentage is much lower than that of the amino acid, possibly indicating that it takes part in fewer transactions particularly of a synthetic nature; there is no evidence that it can be utilised for synthetic purposes beyond the feeding experiments previously described, in which urea caused a marked retention of nitrogen, and the outstanding feature of these investigations was the inability of the amide to act as an adequate substitute for protein.

The percentage of ammonia, the simplest form of tissue nitrogen, is again very much smaller than that of the amide. Does this small quantity of ammonia indicate participation in a very small number of metabolic transactions or does it represent the minimum necessary for purposes of neutralisation, *i.e.* to regulate the reaction of the tissues? There is no doubt that ammonia is mainly derived from protein degradation and the evidence of its resynthesis into protein in the body, as we have seen, is certainly not of a nature to suggest that such a process occurs to any extent under normal circumstances.

It will be shown later that there is a higher percentage of amino acid and of amide nitrogen in the liver than in the muscle, the reason for which may be that a larger proportion of the amino acid of the food is metabolised in the former than in the latter.

These three forms of nitrogen are so readily washed out of the tissues that one cannot imagine them to be in chemical union with any of the cell constituents. The same consideration applies to the fixation by the tissues of these same nitrogenous bodies when introduced directly into the blood stream. They are so easily extracted that the nature of their union with the cell seems much more likely to be physical than chemical. The process of adsorption offers at once the simplest and most feasible explanation of the bond, and the release of the adsorbed substance would immediately follow on lowering the concentration of the solution in the process of extraction. Bayliss [1915] points out that adsorption is a necessary preliminary to many chemical reactions and certainly in the body with its colloidal solutions there must be an infinite number of chemical processes which are initiated in this way.

Composition of Normal Tissues.

When the present investigation was started four years ago the interest was focused on the capacity of the liver to fix and absorb ammonia after its introduction into the blood stream, and on the question of whether the ammonia content of the tissues was affected by different diets. It was hoped by adopting the following method that the same set of experiments would throw light on both points. Previous to the actual experiment the dogs were fed on two diets which differed as widely as possible in their nitrogen content, the one consisting of lard and tapioca and the other of meat with or without an addition of dog biscuit. It was expected that a comparison of the initial tissue analyses in the case of animals fed on the two different diets would furnish data as to the part played by diet in the ammonia content of the tissues and that a comparison of the tissue analyses after the injection would determine the changes in the ammonia content caused by the injection. The actual experiment was carried out under complete ether narcosis; after one or more lobes of the liver had been ligatured off for the initial tissue analysis, the ammonium salt was injected intravenously. The animal was killed by bleeding shortly after the cessation of the injection and the remaining lobes of the liver were then utilised for the determination of the final ammonia content.

After the first few experiments the research began to widen out, muscle tissue was analysed as well as liver, and in addition to the ammonia, total soluble or non-protein nitrogen, amino acid and amide nitrogen were determined with a view to finding out the initial nitrogen distribution in the tissues and the effect produced on it by intravenous injection. An investigation of the disappearance of ammonia from the blood was quickly given up because of the impossibility of carrying out so many estimations single-handed, and for the same reason an analysis of the intestinal mucosa was only carried out in four of the earlier experiments. A further question arose after the first seven experiments, viz. was there any evidence of protein synthesis after the intravenous injection of ammonia, and this entailed a determination of the total nitrogen of the tissues before and after injection together with an estimation of their water content.

As the initial variations in the nitrogen content of the tissues were found to be very wide, the attempt to determine the influence of diet upon it was abandoned after the fifteenth experiment (dog G); the variations up to this time had no definite relation to diet and were therefore judged to be physiological, and the correctness of this deduction was confirmed by the subsequent work in which the variations were about equally wide although a uniform diet of porridge and milk was employed[1].

For purposes of comparison and control a short series of experiments was carried out to ascertain whether the intravenous injection of physiological salt solution caused any change in the nitrogen content and its distribution or in the water content of the tissues. In the later experiments the effect of injecting glycocoll and urea was also investigated. Unfortunately the unavoidable interruption of the research led to its extension over more than two years, and as the later experiments were planned in the light of the earlier work, they naturally furnished on the whole more concordant results and required fewer repetitions. In dealing with the methods employed it is only necessary to describe the procedure in the later experiments as any modifications introduced have only been slight. The results of the experiments are all tabulated in Tables I–XV.

Technique and methods.

After a preliminary hypodermic injection of morphia the animal was anaesthetised with chloroform followed by ether. The jugular vein was first exposed and ligatures were placed in position, the left common iliac artery and vein were then ligatured to facilitate amputation of the thigh, which was performed as high up as possible in order to furnish the maximum amount of muscle tissue for analysis; the bleeding was completely controlled by including all the muscles above the seat of amputation in three interlaced tape ligatures. If the bladder contained any urine it was emptied by direct pressure or failing this by an incision, which was then closed by a purse-string suture. Two or even three lobes of the liver were then ligatured with tape and removed, and immediately after this the cannula was inserted into the jugular vein and the intravenous injection was started. The solution was warmed before being placed in the burette and was run in continuously in the case of the urea and glycocoll but the injection of the ammonia had to be frequently interrupted because of its toxic effect. The animal was killed by bleeding about ten minutes after the cessation of the injection; a few exceptions in the case of some of the ammonia experiments were due to the death of the animal from ammonia poisoning during the injection[1]. After the death of the animal the rest of the liver was at once removed and the other thigh was amputated. If the bladder contained any urine it was ligatured off and removed to prevent any loss of the contents. The blood was carefully and thoroughly squeezed out of each lobe of the liver, immediately after its removal, by firm pressure with the fingers; three small portions from about 0·2 to 0·3 g. of tissue were quickly excised and placed in weighing bottles of known weight, the latter were immediately replaced in the desiccator and were weighed as soon as possible. When practicable 100 g. of liver were used for the determination of the non-protein nitrogen but sometimes a much smaller quantity only was available, viz. 50 to 60 g. After weighing, the tissue was plunged into 100 cc. of acidified boiling water and after being cut up in the fluid into small pieces with scissors in order to ensure complete coagulation of the protein it was boiled for five minutes. (This particular modification of the earlier technique, in which the tissue was boiled in acidified water after being ground up with glass, was adopted from the work of van Slyke.) The muscle tissue was treated in the same way.

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[1] This does not imply that diet has no influence on the composition of the tissues but that no appreciable change could be attributed to the diet in these particular experiments.

The further treatment of the tissue set aside for the determination of the non-protein nitrogen and its constituents was as follows: the liquid was poured off into a porcelain basin and the tissue was pounded with ground glass in a mortar and was then transferred to the liquid in the basin with an additional 100 cc. of water, the whole was then boiled with constant stirring to prevent bumping. After cooling the precipitate and filtrate were transferred to a 500 cc. cylinder and the volume was made up to 400 cc.

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[1] One of the five dogs destined for the injection of normal saline solution died before the injection started apparently from an overdose of the anaesthetic, the tissues were worked up in the usual way however, duplicate samples of liver and muscle being taken for the determination of the soluble nitrogen and its constituents. Although both values have been given in the tables only the figures in the column of initial values have been included in the calculation of the various averages relating to the whole series or to the particular group of experiments.

Aliquot portions of the filtrate were taken for the various analyses, 25 cc. for the non-protein nitrogen and 50 cc. for the ammonia, amide and amino nitrogen. Duplicate analyses of the ammonia and amide nitrogen could not be carried out owing to the number of determinations necessary, but a sufficient check was established by the repetition of the experiments. The analytical methods employed were the same as those used in the previous work [Bostock, 1912, 1] on the nitrogen distribution in autolysis and were as follows: non-protein nitrogen by the Kjeldahl method, ammonia by the Kruger-Reich-Schittenhelm method, amide by a modification of the Benedict-Gephart autoclave method and amino acid by the Henriques-Sörensen method, the initial neutralisation being carried out with litmus as indicator.

The total nitrogen was at first estimated on the moist tissue in duplicate, in all the later work, however, it was estimated in triplicate on the dried tissue; the same samples having been previously weighed in the moist condition served also for the determination of the percentage of dried matter in the fresh tissue, drying being carried out at a temperature of from 100° to 103°.

There is no record of any observations dealing primarily with variations in the composition of the normal tissues and little attention has been drawn to the fact that very considerable differences do occur, although the initial tissue analysis in certain types of experiment furnishes some data on the subject. Emmet and Carroll [1911] however have pointed out some most interesting facts with regard to the physical constants of fat; there was no change produced in the fat of an animal by feeding it with different amounts of protein but great differences were found in the fat of the same animal in different regions of the body, *e.g.* the melting point was 34·1° for the back fat and 45·7° for the kidney fat. Van Slyke [1913, 2] drew attention to the fact that the amino acid content of the gracilis muscle was normally about 10 to 20 mg. less per 100 g. than that of the triceps, and elsewhere [1913, 1] he stated that when different samples of an organ such as the liver were taken as duplicate samples or when symmetrically placed organs such as the right and left kidney gracilis were used, a variation up to 10 per cent. of the amount of amino nitrogen determined must be expected. The following instances may be quoted: lobe (1) 34 mg., lobe (2) 29 mg. [van Slyke and Meyer, 1913, 1], and again, lobe (1) 59 mg., lobe (2) 55 mg., lobe (3) 64 mg. [van Slyke and Meyer, 1913, 3].

That variations exist in the non-protein nitrogen of the blood in different individuals of the same species may be seen from an examination of Folin's figures; in the first series of experiments [Folin and Denis, 1912, 1] these range from 30 to 40 mg. per 100 cc. of portal blood, while in a later series [Folin and Denis, 1912, 3] the extent of the variation is even greater, the limits per 100 cc. of blood being 29 and 67 mg.; the minimum values it may be mentioned in the last named experiments are unmistakably due to the diet.

The nitrogen of the tissues is of course mainly in the form of coagulable or protein nitrogen but a small percentage can be washed out of the tissue, which is neither coagulable by heat nor affected by the ordinary precipitants of protein. The amount of this soluble nitrogen varies slightly according to the method used, in the case of that adopted in the present research it amounts to about 11 per cent. of the total nitrogen. The main constituents of the soluble or non-protein nitrogen are the amino acid, amide and ammonia nitrogen and together they constitute about 60 per cent. of the soluble nitrogen.

The term *nitrogen distribution* in this paper is used to designate the actual and relative amounts of these different forms of nitrogen, and the present series of experiments will furnish data regarding the particular arrangement existing in different tissues and the changes produced by the intravenous introduction of various forms of nitrogen. The variations which occur in different individuals in the amount of the various nitrogenous bodies and in the relations existing between some of these may be conveniently described under the heading of *physiological variation*, and it will be necessary to consider its limits before proceeding to the study of the effects produced on the nitrogen distribution in the tissues by the introduction of different types of nitrogen into the blood stream.

The difference between the maximum and minimum values in a given series of figures furnishes the total variation for the series and if this figure be halved the total ± variation is obtained; the significance of the variation can only be accurately gauged, however, by expressing it as a percentage of the average value for the whole series of figures under consideration and such a procedure also renders it available for purposes of comparison. This method of treating the total ± variation has been adopted in the present paper. For example, the maximum and minimum values for the series of figures 4, 3 and 2 are 4 and 2 respectively, the total variation is 2 and the total ± variation is ± 1; the mean of the series is 3 and therefore the percentage variation is ± 33·3 per cent. $\left(\frac{1 \times 100}{3}\right)$.

In the case of figures taken from the work of other investigators, the mean of the maximum and the minimum values has been substituted for the mean of the whole series as it was more readily arrived at and did not seriously affect the resulting value.

The physiological variation in the normal tissues will be considered under the following headings:

1. Percentage of dried material in 100 g. fresh tissue.
2. Total nitrogen in 100 g. fresh and dried tissue.
3. Total insoluble or protein nitrogen in 100 g. fresh and dried tissue.
4. Total soluble or non-protein nitrogen in 100 g. fresh tissue.
5. Relationship of the total protein and non-protein nitrogen to the total nitrogen.
6. Amino acid nitrogen in 100 g. fresh tissue.
7. Amide nitrogen in 100 g. fresh tissue.
8. Ammonia nitrogen in 100 g. fresh tissue.

1. *Physiological variation in the percentage of dried tissue in* 100 *g. fresh tissue.*

It is evident that the percentage of moisture in the tissues will affect the figures for the nitrogen content of the fresh tissue and it might conceivably be responsible for the variations in the latter. It will be seen from Table I that there are considerable differences in the percentage of dried matter in the fresh tissue of both liver and muscle in different individuals. The limits for the liver are 28·4 per cent. and 21·9 per cent. giving a total variation of ± 3·2 or of ± 12·1 per cent., which is less than the percentage variation in the values for every variety of nitrogen in the fresh liver, and therefore the amount of moisture cannot be the determining factor in variations of the nitrogen content. The average value for the whole series is 26·64 per cent. and may be compared with the average for Grund's whole series which is 25·5 per cent. In the case of the muscle the limits are 28·4 per cent. and 22·3 per cent. giving the same percentage variation of ± 12·1 per cent. as in the liver; here again the variation in the moisture is smaller than the variation in any of the forms of nitrogen present in the fresh muscle. The average value for the muscle is 24·8 per cent. and is slightly higher than Grund's average, which works out at 23·9 per cent. It may be mentioned that it by no means always follows that a high percentage of moisture in the liver is associated with the same condition in the muscle; in about 47 per cent. of the cases the value is above the average in the one and below it in the other tissue and neither the maximum nor minimum value for the liver is associated with the corresponding maximum or minimum value in the muscle.

TABLE I.

Giving percentage of dried matter in 100 *g. fresh tissue before and after injection.*

Experiments		Liver		Muscle	
		Before	After	Before	After
Early ammonia	*	28·41	25·54	24·59	24·57
,,	A	26·1	26·2	28·4	28·4
,,	B	26·3	25·9	27·7	25·9
,,	C	27·72	25·95	26·25	25·55
,,	D	26·92	24·33	23·8	23·78
,,	E	27·25	26·98	24·32	23·11
,,	F	27·71	23·87	24·46	23·68
,,	G	27·68	25·92	26·23	25·17
Last ammonia	I	21·9	21·7	23·1	23·5
,,	II	26·7	24·5	22·3	22·6
Sodium chloride	I	27·71	28·43	26·66	26·67
,,	II	27·97	26·23	26·04	26·88
,,	III	28·07	24·68	24·49	25·55
,,	IV	26·38	21·77	24·89	25·14
,,	V	26·82	24·61	25·06	24·73
Glycocoll	I	24·73	22·14	23·69	23·49
,,	II	24·38	24·57	24·61	23·33
,,	III	26·94	22·15	24·39	24·11
,,	IV	26·8	24·9	24·17	24·7
,,	V	27·61	25·40	25·11	25·66
,,	VI	26·64	24·34	24·46	24·36
Urea	I	26·5	27·1	22·8	23·5
,,	II	25·5	25·7	25·05	24·62
Average for whole series		26·64	25·05	24·89	24·72

2. *Total nitrogen calculated on the fresh tissue.*

These figures will be found in Table II. The average value for 100 g. liver is 3·064 g. and there is a total variation of ± 0·443, or of ± 14·4 per cent. The average value for the muscle is slightly higher and is 3·397 g., with a total variation of ± 0·516 g. or of ± 15·1 per cent. Neither the maximum nor the minimum values for the liver are found in conjunction with the maximum or minimum values for the muscle, and the lack of any close correspondence between the relative amounts of the total nitrogen of the two tissues is borne out by the fact that in 38 per cent. of the experiments a high value in the one tissue is accompanied by a low value in the other. Buglia and Constantino [1913] give 3·240 g. and 3·390 g. for the total nitrogen of the muscle of two fed dogs; these figures coincide closely with the average value given above. Diesselhorst's figures [1911] in the case of two dogs are as follows:

Rice diet: Dog 1, 3·08 g. ... Dog 2, 3·21 g.
Meat diet: Dog 1, 3·27 g. ... Dog 2, 3·46 g.

TABLE II.

Giving the total nitrogen in g. in 100 *g. fresh and dried tissue before and after injection.*

		Fresh liver		Dried liver		Fresh muscle		Dried muscle	
Experiments		Before	After	Before	After	Before	After	Before	After
Early ammonia	*	3·192	3·287	11·23	12·88	3·599	3·631	14·63	14·77
"	A	2·990	2·890	11·45	11·03	4·081	3·318	14·36	11·68
"	B	2·891	2·289	10·99	8·84	3·346	3·069	12·08	11·85
"	C	2·590	2·947	9·34	11·35	3·365	3·328	12·81	13·02
"	D	3·165	2·029	11·75	8·33	3·302	3·252	13·87	13·67
"	E	3·058	3·370	11·22	12·49	3·269	3·150	13·44	13·63
"	F	2·793	2·933	10·08	12·28	3·328	3·188	13·60	13·46
"	G	2·591	2·950	9·36	11·38	3·366	3·328	12·83	13·22
Last ammonia	I	3·128	3·011	12·42	13·25	3·247	3·351	13·48	14·04
"	II	2·918	3·037	10·92	12·39	3·048	3·112	13·62	13·69
Sodium chloride	I	3·251	3·489	11·73	12·27	3·482	3·471	13·05	13·01
"	II	2·697	2·859	9·64	10·90	—	—	—	—
"	III	3·009	2·952	10·71	11·95	3·277	3·312	13·37	12·96
"	IV	3·185	2·730	12·02	12·52	3·492	3·485	14·02	13·90
"	V	3·043	3·022	11·59	12·42	3·409	3·398	13·60	13·79
Glycocoll	I	3·070	2·970	12·41	13·41	3·290	3·352	13·88	14·27
"	II	3·202	3·333	13·13	13·56	3·494	3·606	14·19	15·45
"	III	3·107	2·746	11·53	12·39	3·448	3·579	14·13	14·84
"	IV	3·467	3·209	12·93	12·87	3·381	4·131	14·19	16·67
"	V	2·960	3·046	10·71	11·96	3·504	3·597	13·91	14·01
"	VI	3·476	3·299	13·04	13·55	—	—	—	—
Urea	I	3·419	3·578	12·88	13·17	3·213	3·326	14·04	14·13
"	II	3·271	3·322	12·82	12·88	3·409	3·426	13·60	13·91
Average for whole series		3·064	3·012	11·47	12·07	3·397	3·396	13·65	13·80

Calculated on 100 g. dried tissue the average value for the liver is 11·47 g. with a total variation of ± 1·89 g. or of ± 16·4 per cent. The minimum value coincides with the minimum value for the fresh tissue and Table III shows that there is a general correspondence between the values for the moist and dried tissue in the different groups of experiments, a high or low value in the one being accompanied by a correspondingly high or low value in the other with the exception of the last ammonia group. The average value for muscle is 13·65 g. with a total variation of ± 1·275 g. or of ± 9·3 per cent.

Buglia and Constantino's values for the dried muscle are 14·08 g. and 13·87 g. and those of Diesselhorst are:

Rice diet: Dog 1, 12·82 g. ... Dog 2, 11·44 g.
Meat diet: Dog 1, 11·54 g. ... Dog 2, 12·88 g.

The maximum and minimum values for the dried muscle do not coincide with the corresponding values for the moist muscle and this lack of concordance is evident in two out of the five groups of experiments in Table III, where a high value in the dried tissue is associated with a low value in the fresh and *vice versa*.

TABLE III.

Average value in g. for the different groups of experiments of the total nitrogen *in* 100 *g. moist and dried tissue before and after injection.*

	Fresh liver		Dried liver		Fresh muscle		Dried muscle	
Experiments	Before	After	Before	After	Before	After	Before	After
Early ammonia ...	2·908	2·836	10·67	11·07	3·407	3·283	13·40	13·16
Last ammonia ...	3·023	3·024	11·67	12·82	3·147	3·231	13·55	13·86
Sodium chloride ...	3·000	3·080	11·41	11·88	3·389	3·393	13·34	13·25
Glycocoll ...	3·214	3·100	12·29	12·95	3·423	3·653	14·06	15·04
Urea ...	3·345	3·450	12·85	13·0	3·311	3·376	13·82	14·02
Mean value for the whole series	3·064	3·012	11·47	12·07	3·397	3·396	13·65	13·80

There is a rather closer agreement between the relative amounts of the total nitrogen of the liver and muscle than was found in the case of the total nitrogen calculated on the moist tissue, for in 33 per cent. only of the experiments is a relatively high value in the one tissue associated with a relatively low value in the other. In comparing the percentage variation, it will be seen that it is greater in the case of the dried liver than in that of the fresh organ but the reverse is true of the muscle, showing that in the latter case the water content of the tissue is responsible for some of the variation found in the analysis of the fresh material.

3. *The total insoluble or protein nitrogen.*

The figures for the insoluble nitrogen are obtained by subtracting the total soluble nitrogen from the total nitrogen, the average value for 100 g. liver is 2·772 g. and the total variation is ± 0·398 g. or ± 14·3 per cent. The average value for the muscle is 3·018 g. with a total variation of ± 0·528 g. or ± 17·4 per cent. The maximum value for the one tissue does not coincide with the maximum for the other and the same holds good of the minimum values; in 55 per cent. of the experiments the value is above or below the average respectively in both liver and muscle while in the remaining 45 per cent. a high value in the one is associated with a low value in the other.

TABLE IV.

Total insoluble or protein nitrogen in g. in 100 g. moist and dried tissue respectively before and after injection.

		Fresh tissue				Dried tissue			
		Liver		Muscle		Liver		Muscle	
Experiments		Before	After	Before	After	Before	After	Before	After
Early ammonia	*	2·809	2·934	3·239	3·264	9·88	11·5	13·7	13·2
,,	A	2·778	2·653	3·727	2·971	10·63	10·25	13·08	10·4
,,	B	2·656	2·037	2·963	2·734	10·48	7·8	10·6	10·6
,,	C	2·374	2·681	2·973	2·943	8·70	10·2	11·3	11·5
,,	E	2·833	3·145	2·905	2·789	10·48	11·5	11·8	12·0
,,	F	2·577	2·695	2·986	2·845	9·35	11·2	12·2	12·0
,,	G	2·425	2·759	3·052	3·003	9·52	10·6	11·6	11·9
Last ammonia	I	2·885	2·765	2·852	2·948	13·80	12·7	12·4	12·5
,,	II	2·554	2·667	2·670	2·726	9·56	10·8	11·9	12·0
Sodium chloride	I	2·965	3·183	3·100	3·088	10·7	11·1	11·8	11·2
,,	II	2·426	2·581	—	—	8·7	9·8	—	—
,,	III	2·654	2·571	2·951	2·967	9·44	10·4	11·8	11·6
,,	IV	2·830	2·409	3·085	3·102	10·76	11·0	12·4	12·0
,,	V	2·774	2·726	3·061	3·055	10·4	11·0	12·2	12·1
Glycocoll	I	2·677	2·615	2·944	2·988	10·8	11·7	12·3	12·7
,,	II	2·939	3·020	3·110	3·229	12·0	12·2	12·6	12·9
,,	III	2·742	2·422	3·036	3·159	10·6	9·7	12·3	12·8
,,	IV	3·147	2·830	3·011	3·738	11·75	11·3	12·4	15·1
,,	V	2·637	2·665	3·132	3·202	10·36	10·4	12·3	12·5
,,	VI	3·171	2·937	—	—	12·33	12·0	—	—
Urea	I	3·085	3·191	2·803	2·855	11·62	11·7	12·3	12·1
,,	II	2·980	3·005	3·016	2·987	11·7	11·2	11·9	12·1
Average for whole series		2·772	2·701	3·018	3·013	10·4	10·8	12·0	12·1

The average value for the liver is smaller than for the muscle and in only 15 per cent. of the experiments is the reverse condition found. Grund's [1910] figures for the protein nitrogen are slightly lower than mine; they vary between 2·2 g. and 2·5 g. for the liver and between 2·5 g. and 2·8 g. for the muscle in the case of his six fed dogs.

It is of interest to note that the percentage variation of the insoluble nitrogen is much smaller than that of the soluble or non-protein nitrogen. Calculated on the dried tissue the average for the liver is 10·4 g. with a variation of ± 2·55 g. or ± 24·5 per cent. For the muscle the average is 12·0 g. with a variation of ± 1·55 g. or ± 12·9 per cent. In the case of the liver the variation on the dried weight is considerably greater than on the fresh tissue, which proves conclusively that the main source of the variation does not lie in the percentage of moisture in the tissue. There is only a single exception, namely Experiment ammonia I, to the rule that the protein nitrogen of the dried liver is smaller than that of the muscle.

4. *Total soluble or non-protein nitrogen.*

The figures for the total soluble nitrogen of the fresh tissue will be found in Table V, and glancing at them one is at once struck by the very varying amounts present in the livers of different individuals; while the average for the whole series is 292 mg. in 100 g. fresh tissue there is a total variation of ± 144·5 mg. or of ± 49·4 per cent. The average for the muscle is 379 mg. with a total variation of ± 86·5 mg. or of ± 21·6 per cent. Buglia and Constantino [1913] found almost identical values for the non-protein nitrogen in the case of two dogs, the actual figures being 370 mg. and 390 mg. in 100 g. muscle. Folin's figures [Folin and Denis, 1912, 3] for muscle show a variation of ± 31 per cent. but in this case the diet is certainly responsible for the minimum values. The figures of Fiske and Sumner [1914], who used the methods of Folin, show a total variation of ± 55·5 mg. or of ± 21 per cent. The actual values for the non-protein nitrogen are lower than in my experiments, due probably to the different species of animal and the different method employed. Although in the present research the highest values for the liver and muscle occurred in the same dog and the lowest values were similarly found in one and the same animal, a relatively high nitrogen content in one tissue was by no means always accompanied by high content in the other, for in 50 per cent. of the cases a value above the average in one tissue is associated with a value below the average in the other.

5. *Relationship of the protein and non-protein nitrogen to the total nitrogen.*

The relation of the protein and non-protein nitrogen to the total nitrogen will be found in Tables VI and VII expressed as a percentage of the latter. The average ratio of the non-protein nitrogen to the total nitrogen in the liver for the whole series is 9·5 per cent. with a total variation of ± 3·19 or ± 33·5 per cent., the average for the muscle is 11·1 per cent. with a total variation of ± 2·01 or of ± 18·1 per cent.; in this relation therefore the limits of variation are also narrower in the case of the muscle than of the liver. Although the average value is rather smaller in the case of the liver than of

TABLE V.

Giving the non-protein, amino acid, amide and ammonia nitrogen in mg. per 100 *g. fresh tissue before and after injection.*

		Liver								Muscle							
		Total soluble N		Amino acid N		Amide N		Ammonia N		Total soluble N		Amino acid N		Amide N		Ammonia N	
Experiments		Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Early ammonia	I	—	—	—	—	—	—	11·0	14·9	—	—	—	—	—	—	—	—
,,	II	—	240	—	—	—	—	14·3	27·5	—	—	—	—	—	—	—	—
,,	III	313	358	—	—	48·7	52·9	10·0	11·4	436	403	—	—	46·4	44·7	13·4	14·0
,,	IV	261	239	—	—	24·6	21·8	7·0	20·1	324	347	—	—	23·5	27·3	10·0	11·2
,,	V	257	263	—	—	27·4	38·0	10·6	8·9	397	380	—	—	32·7	31·9	12·0	12·6
,,	VII	445	414	72·9	71·3	48·6	58·2	9·6	11·6	487	456	55·4	52·0	34·9	37·0	9·8	12·8
,,	VIII	350	327	52·0	57·7	57·2	58·4	3·2	5·6	368	385	39·7	33·4	43·3	41·9	8·4	13·1
,,	*	383	359	—	—	—	—	—	—	360	367	—	—	—	—	—	—
,,	A	212	237	78·3	72·4	29·4	37·2	8·3	11·4	354	347	75·3	68·6	33·1	29·6	10·8	8·4
,,	B	235	252	70·7	73·0	27·7	39·1	5·6	9·5	383	335	74·0	64·8	31·6	31·3	9·2	14·2
,,	C	216	266	54·1	62·2	33·4	37·6	5·6	21·7	392	385	63·5	69·5	36·6	37·8	9·2	10·9
,,	D	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
,,	E	225	225	58·0	47·9	33·5	40·9	7·0	17·0	364	361	66·0	56·6	35·0	37·0	9·3	17·6
,,	F	216	238	**76·0**	**72·4** = amino acid + ammonia N					342	343	**91·0**	**90·2** = amino acid + ammonia N				
,,	G	166	191	—	—	—	—	—	—	314	325	—	—	—	—	—	—
Last ammonia	I	243	246	76·1	65·2	37·0	39·8	5·6	18·9	395	403	89·8	87·5	36·9	43·1	9·5	14·0
,,	II	364	370	73·2	67·1	50·7	62·8	5·2	26·4	378	386	85·8	84·2	53·1	57·8	12·1	14·9
,,	III	236	240	63·3	42·0	43·2	40·1	5·0	37·5	379	407	72·1	76·8	32·2	42·7	18·9	20·3
Sodium chloride	I	286	296	72·6	71·8	—	—	10·7	12·9	382	383	—	—	—	—	14·9	14·9
,,	II	271	278	—	—	—	—	4·4	7·8	378	371	71·2	70·9	—	—	7·8	8·9
,,	III	355	381	100·7	107	42·6	43·1	13·3	10·1	326	345	81·8	77·4	36·1	37·3	9·9	9·9
,,	IV	355	321	98·8	80·5	—	—	7·0	7·0	407	383	73·3	80·9	38·0	34·7	9·5	10·0
,,	V	269	296	78·6	84·2	41·4	41·4	6·7	6·7	348	343	83·8	82·0	30·1	29·4	9·8	9·8
Glycocoll	I	393	355	**74·2**	**110·6** = amino acid + ammonia N				—	346	364	—	—	—	—	—	—
,,	II	263	313	67·0	80·8	—	—	13·3	22·5	384	377	—	—	—	—	—	—
,,	III	355	324	96·5	116·6	—	—	5·6	12·8	412	420	82·4	82·3	—	—	10·6	10·0
,,	IV	320	407 351	83·7	156·2 136·3	58·2	64·3 58·8	10·0	17·3 16·2	370	393	89·6	94·7	62·7	63·9	8·4	8·9
,,	V	323	354 408	77·2	138·9 136·3	44·1	51·5 51·3	10·2	10·2 12·8	372	395	96·7	104·2	38·1	40·5	8·2	9·1
,,	VI	305	345 389	68·8	139·7 142·7	50·0	45·3 50·1	7·1	16·6 16·6	399	430	84·1	103·0	43·1	47·5	11·7	11·7
Urea	I	334	387	87·1	76·5	56·6	138·1	9·0	11·6	410	471	85·2	84·9	47·6	98·7	11·9	12·6
,,	II	291	317	72·1	68·4	44·9	115·1	8·6	9·4	393	439	87·8	89·0	44·1	93·0	12·4	12·7
Average for the whole series		292	311	76·3	93·1	41·2	53·6	8·0	15·5	379	383	78·7	79·3	38·6	46·3	10·8	12·3

The figures of Experiment ammonia VIII referring to the goat have not been included in the various averages.

the muscle this condition is reversed in 20 per cent. of the individual experiments. A relatively high or low value in the liver is associated with a correspondingly high or low value in the muscle in 55 per cent. of the experiments.

The average ratio of the protein nitrogen to the total nitrogen in the liver is 90·5 per cent. with the same total variation as in the case of the ratio of non-protein to total nitrogen, which however only amounts to ± 3·5 per cent., when expressed as the percentage variation; this shows in a striking fashion how much more liable to variation is the non-protein nitrogen than the protein nitrogen. The average value for the muscle is 88·9 per cent. with a total variation of ± 2·01 or of ± 2·2 per cent.

TABLE VI.

Giving insoluble or protein nitrogen as a percentage of the total nitrogen before and after injection.

		Liver		Muscle	
Experiments		Before	After	Before	After
Early ammonia	*	88·0	89·26	90·0	90·0
,,	A	92·90	91·80	92	89·6
,,	B	91·88	89·00	88·6	89·0
,,	C	91·67	90·97	88·4	88·4
,,	D	—	—	—	—
,,	E	92·62	93·32	88·8	88·6
,,	F	92·27	91·90	89·7	89·2
,,	G	93·60	93·53	90·6	90·2
Last ammonia	I	92·22	91·82	87·9	88
,,	II	87·52	87·82	87·6	87·6
Sodium chloride	I	91·20	91·24	89	89
,,	II	89·95	90·28	—	—
,,	III	88·20	87·10	90·1	89·6
,,	IV	88·85	88·23	88·3	89·1
,,	V	91·16	90·20	89·8	89·5
Glycocoll	I	87·19	88·04	89·4	89
,,	II	91·79	90·69	89·0	89·6
,,	III	88·25	88·18	88·1	88·3
,,	IV	90·78	88·18	89	90·5
,,	V	89·08	87·50	89·4	89·1
,,	VI	91·22	89·03	—	—
Urea	I	90·23	89·18	87·3	87·9
,,	II	91·12	90·46	88·5	87·2
Average for whole series		90·5	89·7	88·9	88·8

6. *The amino acid nitrogen* (see Tables V and XIII).

The average value for 100 g. fresh liver is 76·3 mg., the total variation being ± 23·3 mg. or ± 30·5 per cent.; a study of the initial values in van Slyke's [van Slyke and Meyer, 1913, 1] experiments reveals a total variation of ± 13·5 mg. or of ± 31 per cent. (calculated as a percentage of the mean (42·5 mg.) of the maximum and miminum values). In one case the amino acid content of two different lobes of the same liver gave a total

TABLE VII.

Giving total soluble or non-protein nitrogen as a percentage of the total nitrogen before and after injection.

Experiments		Liver		Muscle	
		Before	After	Before	After
Early ammonia	*	12·01	10·92	10·02	10·11
„	A	7·11	8·23	8·67	10·46
„	B	8·13	11·01	11·44	10·91
„	C	8·34	12·42	11·65	11·57
„	D	—	—	—	—
„	E	7·35	6·67	11·13	11·45
„	F	7·74	8·13	10·3	10·77
„	G	6·42	6·47	9·34	9·79
Last ammonia	I	7·76	8·16	12·16	12·04
„	II	11·91	11·63	12·4	12·41
Sodium chloride	I	8·79	8·48	10·97	11·03
„	II	10·05	9·72	—	—
„	III	11·83	12·9	9·96	10·41
„	IV	11·15	11·76	11·65	10·9
„	V	8·83	9·79	10·2	10·5
Glycocoll	I	12·80	11·9	10·51	10·85
„	II	8·21	9·39	11·0	10·4
„	III	11·42	11·79	11·94	11·73
„	IV	9·22	10·93 12·7	10·94	9·51
„	V	10·93	11·62 13·41	10·61	10·99
„	VI	8·77	10·45 11·79	—	—
Urea	I	9·76	10·8	12·72	14·16
„	II	8·89	9·55	11·53	12·81
Average for whole series		9·5	10·3	11·1	11·2

variation of 8 per cent. It will be seen that the Sörensen method in my hands gave a rather higher figure than van Slyke found by his method.

The average value for the muscle is 78·7 mg. with a total variation of ± 20·6 mg. or of ± 26·1 per cent. In the case of the gracilis, van Slyke's figures show a total variation of ± 15·5 mg. or of ± 30 per cent.; the extremely interesting fact that the amino acid content of the triceps is usually 10 to 20 mg. higher than that of the gracilis has already been referred to. Buglia and Constantino using the Sörensen method found an amino acid content in the muscle of two fed dogs of 84 mg. and 77 mg. respectively.

In 64 per cent. of the experiments a relatively high or low value in the liver is associated with a correspondingly high or low value in the muscle but neither maximum nor minimum values occur in the same animal. Although the average value for the muscle is slightly higher than that of the liver this condition is reversed in 35 per cent. of the experiments and a similar variability is present in van Slyke's experiments, the amino acid content of the liver being sometimes higher and at other times lower than that of the muscle.

In comparing the values of the amino acid nitrogen with those of the soluble nitrogen, 73 per cent. of the experiments show a relatively high or low value for both forms of nitrogen in the liver although neither the maximum nor the minimum values occur in the same dog; 47 per cent. of the experiments show a relatively high or low value for both forms of nitrogen in the case of the muscle.

7. *Amide nitrogen* (see Tables V and XIII).

The average value for the amide nitrogen in 100 g. fresh liver is 41·2 mg. and the total variation is ± 16·8 mg. or ± 40·7 per cent.; for the muscle the average is slightly smaller, namely, 38·6 mg. with a total variation of ±19·6 mg. or ± 50·7 per cent. The maximum values for both tissues occur in the same dog and the same holds good for the minimum values; in only 28 per cent. of the experiments is a relatively high value in the one tissue associated with a relatively low value in the other. In Folin's first paper there is a total variation in the urea values for the muscle of ± 11·5 mg. or of ± 29 per cent. (calculated as a percentage of the mean of the maximum and minimum values), in his third paper the percentage amounts to ± 44 per cent. but here also as in the case of the non-protein nitrogen the influence of diet is unmistakable, the minimum values occurring in cats fed on a rice and cream diet. In Fiske and Sumner's [1914] experiments the urea values for the muscle range from 14 mg. to 82 mg. which gives a total variation of ± 70 per cent.

Although the average figure for the liver is slightly higher than for the muscle the reverse condition is present in 39 per cent. of the experiments. In the liver there is quite a close general correspondence between the values for the total soluble nitrogen and the amide nitrogen; although neither maximum nor minimum values are found in the same animal, in 83 per cent. of the cases a relatively high value for the one form of nitrogen is associated with a correspondingly high value for the other form and *vice versa*. In the muscle on the other hand such a correspondence is only present in 55 per cent. of the experiments.

8. *Ammonia nitrogen* (see Tables V and XIII).

The average amount of ammonia nitrogen present in 100 g. fresh liver is 8·0 mg., the total variation is ± 4·6 mg. or ± 57·9 per cent.; the average for the muscle is rather higher, 10·8 mg. with a total variation of ± 5·5 mg. or of ± 51·2 per cent. The minimum values occur in the same dog but the maximum values do not coincide; in 57 per cent. of the animals the values are correspondingly high or low in both liver and muscle.

With regard to the relation of ammonia to the total soluble nitrogen, the value is correspondingly high or low in 56 per cent. of the experiments in the case of the liver and in 66 per cent. in that of the muscle.

Percentage distribution of the amino, amide and ammonia nitrogen in the total soluble nitrogen.

An examination of the average initial values for the whole series shows clearly that the percentage of the different forms of non-protein nitrogen in the total soluble nitrogen differs quite definitely in the liver and muscle; the percentage of amino acid and of amide nitrogen is considerably greater in the liver than in the muscle, and on the other hand the percentage of undetermined nitrogen in the latter is higher than in the liver; there is therefore a characteristic distribution of the constituents of the non-protein nitrogen for each tissue.

TABLE VIII.

Average values for the different groups of experiments expressed as a percentage of total soluble or non-protein nitrogen.

	Liver						Muscle					
	Percentage of Amino acid N		Percentage of Amide N		Percentage of Ammonia N		Percentage of Amino acid N		Percentage of Amide N		Percentage of Ammonia N	
Experiments	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Early ammonia	23·4	22·8	13·3	15·2	3·2	3·7	16·5	15·5	9·3	9·6	3·2	3·4
Last ammonia	24·6	21·8	14·4	16·6	1·7	8·9	22·7	21·7	11·6	12·7	3·4	4·1
Sodium chloride	28·6	27·3	13·7	13·4	2·7	2·8	21·0	21·3	9·4	9·2	2·7	2·9
Glycocoll	24·1	36·3	15·5	14·8	2·8	4·3	23·2	24·2	12·6	12·7	2·5	2·4
Urea	25·5	20·5	16·2	35·9	2·6	4·0	21·5	19·0	11·4	21·1	2·5	2·7
Average value for whole series	26·1	29·9	14·1	17·2	2·7	4·9	20·7	20·7	10·1	12·0	2·8	3·2

In this connection attention may be directed to the influence exerted by the presence of acid or alkali on the characteristic nitrogen distribution of the autolysing liver in vitro [Bostock, 1912, 2]. The addition of alkali causes the production of a higher percentage of ammonia and of a lower percentage of amide and amino acid nitrogen, whereas the addition of acid brings about a diminution in the percentage of ammonia and an increase in the percentage of amide and amino acid nitrogen. Whether the preponderance of bases in muscle tissue is in any way responsible for the characteristic distribution of nitrogen in this tissue would be difficult to determine, but it approximates more closely to the distribution in autolysing liver in presence of alkali than to that of normal autolysing liver, while it differs from autolysing liver in presence of acid more than from normal autolysing liver.

EFFECT OF INJECTION OF VARIOUS SUBSTANCES ON TISSUE COMPOSITION.

Having studied the variations in the constituents of the nitrogenous material of the tissues which occur in the liver and muscle of normal dogs we may pass to a consideration of the effects produced by the introduction of physiological salt solution and of different nitrogen-containing solutions. The same aspects of tissue analysis will be dealt with as in the section on physiological variation and in a similar order with the exception of the total nitrogen, which has not been treated by itself but only in relation to its constituents.

Table IX contains data regarding the amount of the particular substances and the volume of fluid injected together with the amount of nitrogen introduced per kilo. body weight in the different experiments. It will be seen that in all the earlier work, including all the experiments with sodium chloride and the first three with glycocoll and the ammonia experiments except the last three, dilute solutions practically isotonic with physiological salt solution were used because it was thought that this would interfere less with the normal water content of the tissues. In the later experiments much more concentrated solutions were used and with more striking results as both the glycocoll and ammonia experiments show. It should be mentioned that in the last ammonia experiment 90 cc. in all of a 10 per cent. solution of glucose were introduced alternately with the solution of ammonia. With regard to the urine secreted during the operation, as a rule in the ammonia experiments little or no urine was found in the bladder on the death of the animal, so that the amount of ammonia excreted by this

TABLE IX.

Giving all the experiments and data as to weight of the animal and amount of nitrogen per kilo. body weight contained in the injected fluid, etc., etc.

Experiments		Kilos. body weight	g. of substance injected	g. N in amount injected	g. N per kilo. body wt.	Duration of injection in minutes	Strength of solution	cc. fluid injected	Diet previous to experiment	Remarks
Early ammonia	I	21	7	1·673	0·079	5	isotonic	—	—	—
,,	II	7	2·25	0·537	0·076	25	,,	—	milk and tapioca	died during injection
,,	III	20	4·28	1·022	0·051	33	,,	—	meat	—
,,	IV	6·7	2·98	0·712	0·106	30	,,	—	milk and tapioca	died during injection
,,	V	7	4·79	1·114	—	120	,,	—	milk, lard, tapioca	killed 10 min. after injection
,,	VII	11	2·66	0·635	—	48	,,	—	meat	,, ,,
,, (goat)	VIII	—	—	—	—	—	—	—	—	—
,,	*	—	1·97	0·47	—	47	isotonic	197	tapioca, lard	died during injection
,,	A	—	5·4	1·323	—	60	,,	545	—	—
,,	B	15	6	1·47	0·098	75	,,	600	tapioca, lard	—
,,	C	6·65	2	0·49	0·073	30	,,	20·2	meat, dog biscuit	died during injection
,,	D	7·2	3·7	0·906	0·125	50	,,	378	,, ,,	,, ,,
,,	E	8·3	5	1·225	0·147	75	,,	500	,, ,,	—
,,	F	6·6	3·24	0·793	0·120	80	,,	—	,, ,,	—
,,	G	9·7	4·02	0·929	0·095	100	,,	402	,, ,,	—
Last ammonia	I	8·3	5·05	0·901	0·106	72	5 %	101	porridge and milk	died during injection
,,	II	6	4·15	0·74	0·123	63	5 %	83	,, ,,	,, ,,
,,	III	7·2	7·15	2·085	0·28	56	5 %	143	,, ,,	,, ,,
Sodium chloride	I	11·25	—	—	—	80	0·9 % NaCl	710	,, ,,	—
,,	II	—	—	—	—	—	,,	—	,, ,,	—
,,	III	7·65	—	—	—	40	,,	1000	,, ,,	—
,,	IV	12·7	—	—	—	—	—	—	,, ,,	died before injection
,,	V	4·73	—	—	—	30	0·9 % NaCl	550	,, ,,	—
Glycocoll	I	6·22	8	1·49	0·24	60	1·6 %	500	,, ,,	—
,,	II	6·62	12·8	2·39	0·36	90	,,	800	,, ,,	—
,,	III	23·5	16	2·98	0·12	46	,,	1000	,, ,,	—
,,	IV	31·9	30	5·6	0·17	50	6 %	500	,, ,,	—
,,	V	13·2	15	2·8	0·21	31	6 %	250	,, ,,	—
,,	VI	6·5	13·3	2·48	0·38	49	10 %	133	,, ,,	—
Urea	I	8·9	12·55	5·85	0·65	50	5 %	251	,, ,	—
,,	II	6·9	8·05	3·75	0·52	75	5 %	161	,, ,,	—

channel must have been very small. In the glycocoll experiments on the other hand the bladder was quite frequently full at the close of the experiment and the amount of amino acid excreted can be seen in Table X; in no case does it amount to more than 20 per cent. of the total amount injected. A considerable amount of amide nitrogen was excreted in the second urea experiment; unfortunately the urine in the case of the first urea experiment was lost.

TABLE X.

Giving the amount of total nitrogen, amino acid, amide and ammonia nitrogen in mg. excreted in the urine secreted during the period of the injection.

Experiments		Total Nitrogen	Amino acid Nitrogen	Amide Nitrogen	NH_3 Nitrogen
Glycocoll	I	602	304 = amino acid + NH_3 nitrogen		
,,	II	1062	312·6	—	51·8
,,	III	267	14·3	—	11·5
,,	IV	611	355	—	28·5
,,	V	—	456·9	—	23·2
,,	VI	215	158·5	—	6·1
Urea	II	730	—	670	28

If one wishes to produce the maximum fixation by the tissues of the various nitrogen-containing substances introduced into the blood stream, then undoubtedly this is most readily brought about by having a high concentration of the substance in the blood. This requisite concentration is best obtained by the rapid introduction of a concentrated solution of the substance under examination. This is well exemplified in the case of the experiments with urea (see Table V) when the amount injected corresponded to 0·6 and 0·5 g. nitrogen respectively per kilo. body weight.

TABLE XI.

Giving average percentage of dried tissue in 100 g. moist tissue in the different groups of experiments.

	Liver		Muscle	
Experiments	Before	After	Before	After
Early ammonia ...	27·26	25·58	25·71	25·02
Last ammonia ...	24·3	23·1	22·7	23·0
Sodium chloride ...	27·39	25·98	25·56	25·95
Glycocoll ...	26·18	23·91	24·40	24·27
Urea	26·0	26·4	23·9	24·06
Average for whole series	26·64	25·05	24·89	24·72

The effect of intravenous injection on the percentage of dried tissue in fresh liver and muscle (see Tables I and XI). The percentage of dried tissue in the fresh liver after injection is 25·05 and is rather smaller than the initial value, the total variation is ± 3·36 or ± 13·4 per cent., a rather wider range than was found before the injection. In 77 per cent. of the experiments the percentage is smaller after injection than in the initial values, showing that in these cases the injection has increased the water content of the tissue. The average for the muscle is 24·7 per cent. and is practically the same as the initial value, the total variation is ± 2·9 or ± 11·7 per cent. In half the experiments the initial value is greater than the value after injection, in the remaining half this condition is reversed. A study of the individual experiments or groups of experiments shows that the water content of the muscle varies as the result of the injection much less than that of the liver. It is quite evident, however, in both cases that the changes in the water content of these tissues offer a totally inadequate explanation for all the variations in the different forms of nitrogen which have been observed. This is particularly the case in those experiments where definite changes were produced in the various nitrogenous constituents of the total soluble nitrogen by the injection of urea, glycocoll and ammonia.

TABLE XII.

(*a*) *Giving total insoluble or protein nitrogen in g. per* 100 *g. fresh tissue, calculated as the average value for each group of experiments.*

	Liver		Muscle	
Experiments	Before	After	Before	After
8 early ammonia ...	2·636	2·700	3·120	2·935
Last ammonia ...	2·719	2·716	2·761	2·837
Sodium chloride ...	2·729	2·765	3·049	3·036
Glycocoll	2·885	2·748	3·046	3·263
Urea	3·032	3·088	2·909	2·921
Mean for whole series	2·772	2·701	3·018	3·013

(*b*) *Giving total insoluble or protein nitrogen in g. per* 100 *g. dried tissue. calculated as the average value for each group of experiments.*

	Liver		Muscle	
Experiments	Before	After	Before	After
Early ammonia ...	9·669	10·55	12·13	11·73
Last ammonia ...	11·19	11·75	12·16	12·33
Sodium chloride ...	9·965	10·64	11·93	11·70
Glycocoll	11·01	11·49	12·48	12·57
Urea	11·66	11·69	12·17	12·14
Mean for whole series	10·4	10·8	12·0	12·1

The effect of intravenous injection on the protein nitrogen (see Tables IV and XII). A consideration of the amount of nitrogen injected in the form of ammonia, amide or of amino acid shows how useless it would be to expect to find signs of its influence on the protein nitrogen; the maximum amount injected was 0·065 g. per 100 g. of tissue in the case of the first urea dog.

If the assumption be made that the injected nitrogen is converted into protein and that it is fairly evenly distributed throughout the body, as the protein nitrogen content of the liver under normal conditions is about 2·70 per cent. this would only give a possible increase to 2·76 per cent., a change small enough to be within the limits of experimental error. It will be seen from Table XII that the average value for the whole series in the case of both liver and muscle whether calculated on the fresh or the dried tissue is practically identical before and after injection, with the exception of a slight diminution in the fresh liver compared with the initial value. In 57 per cent. of the individual experiments however, the value after injection is greater than the initial value in the liver while in the remaining percentage the reverse is true; the range of variation is ± 21·3 per cent. The initial value in the muscle is slightly less than the subsequent value in 52 per cent. of the cases and the range of the variation is ± 16·7 per cent.

In 90 per cent. of the experiments a high or low initial value in the liver is associated with a correspondingly high or low value after injection, and this holds good in 84 per cent. of the experiments in the case of the muscle.

The effect of injection on the total soluble nitrogen (see Tables V, XIII and XIV). The results of the injection of the various solutions can best be studied in Table XIII where the average values for each different group are gathered together, for, as might have been expected from the study of the physiological variation in the normal tissues, individual variations after injection are very considerable; the general trend of the experiments becomes quite clear when the average of the group of similar experiments is examined. Starting with a consideration of the changes effected in the soluble nitrogen, it will be seen that in the liver there is an increase in every group, not excluding the sodium chloride group, but the only significant increase is in the urea and glycocoll groups, where it amounts to 12 and 10 per cent. respectively calculated as a percentage of the initial value. In the individual experiments there are exceptions to the general rule about the relation of the initial to the subsequent values, 23 per cent. of these show a diminished value after injection. Attention must be directed to the glycocoll experiments IV, V and

TABLE XIII.

Giving the average values for the different groups of experiments. *Figures represent mg. N. in* 100 *g.* moist *tissue.*

	Liver								Muscle							
	Total soluble N		Amino acid N		Amide N		Ammonia N		Total soluble N		Amino acid N		Amide N		Ammonia N	
Experiments	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Early ammonia	274	280	64·3	64·0	36·7	42·6	8·8	10·5	376	369	62·3	57·4	35·2	35·7	12·1	12·6
Last ammonia	303	308	74·6	66·1	43·8	51·3	5·2	**27·6**	386	394	87·8	85·8	45	50·4	13·2	**16·4**
Sodium chloride	306	314	87·6	85·8	42	42·2	8·4	8·9	368	365	77·5	77·8	34·7	33·6	10·3	10·7
Glycocoll	326	360	78·6	**130·9**	50·7	53·5	9·2	15·6	380	396	88·2	**96**	47·9	50·6	9·7	9·9
Urea	312	352	79·6	72·4	50·7	**126·6**	8·3	14·4	401	455	86·5	86·9	45·8	**96·0**	12·1	12·6
Average value for the whole series	292	311	76·3	93·1	41·2	53·6	8·0	15·5	379	383	78·7	79·3	38·6	46·3	10·8	12·3

TABLE XIV.

Giving the average values for the different groups of experiments. *Figures represent g. N. in* 1000 *g.* dried *tissue.*

	Liver								Muscle							
	Total soluble N		Amino acid N		Amide N		Ammonia N		Total soluble N		Amino acid N		Amide N		Ammonia N	
Experiments	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Early ammonia	10·0	10·9	2·35	2·50	1·35	1·67	0·323	0·42	14·6	14·7	2·42	2·38	1·36	1·43	0·469	0·505
Last ammonia	13·6	16·4	3·30	2·86	1·80	2·21	0·220	1·18	17·0	17·1	3·84	3·72	1·98	2·18	0·58	0·715
Sodium chloride	11·2	12·2	3·03	2·95	1·54	1·62	0·306	0·342	14·4	14·1	3·04	3·00	1·36	1·29	0·400	0·41
Glycocoll	12·4	15·0	3·00	5·80	1·94	2·24	0·352	0·650	15·6	16·3	3·60	3·96	1·95	2·06	0·396	0·405
Urea	12·1	13·3	3·03	2·74	1·93	4·75	0·317	0·545	16·7	18·9	3·61	3·60	1·91	4·00	0·425	0·535
Average for whole series	10·9	12·4	2·86	3·65	1·52	2·10	0·300	0·618	15·2	15·4	3·16	3·20	15·5	18·7	0·440	0·495

VI, where after the injection two separate portions of liver were analysed, there is a considerable difference in the figures obtained for the different portions, but this is not surprising in the light of van Slyke's discovery, already referred to, that the amino acid content of different lobes may vary in a very marked degree. The soluble nitrogen of the muscle only shows a definite increase, amounting to 13 per cent. in the urea group; in the sodium chloride and early ammonia groups it is slightly less than the initial value, and this condition is present in 40 per cent. of the individual experiments. It may be observed that when the values are calculated on the dried muscle instead of on the fresh tissue it is only in the sodium chloride group that the initial is greater than the subsequent value, and even here the difference is really negligible. The total variation in the muscle after injection is ± 19 per cent. and is actually smaller than the variation in the initial values.

The effect of intravenous injection on the ratios of non-protein and protein nitrogen respectively to the total nitrogen (see Tables VI and XV). The average value for the ratio of the non-protein nitrogen in the liver after injection is rather higher than the initial value and amounts to 10·3 per cent., the total variation is ± 3·47 or ± 33·6 per cent. In 27 per cent. of the experiments, however, the initial value is the greater of the two and in 4 per cent. it is unaltered, only in the urea and glycocoll groups is the average value for the group definitely increased by the injection.

The average for the muscle is 11·2 per cent. with a total variation of ± 2·32 or of ± 20·7 per cent. although the average after injection is slightly greater than that of the initial value; in 27 per cent. of the experiments this condition is reversed and in 5 per cent. it is unaltered, only in the urea group is there a definite increase due to the injection.

The total variations of the ratios of the protein nitrogen to total nitrogen are of course the same as those of the ratio of the non-protein to total nitrogen, but expressed as percentage variations they only amount to ± 3·8 per cent. for the liver and ± 2·2 per cent. for the muscle and are practically the same as those of the initial values.

Effect of the injections on the amino acid nitrogen (see Tables V and XIII). Turning now to the amino acid nitrogen, it appears that only in the amino acid group is the value for the liver increased after injection, in all the other groups and in 52 per cent. of the individual experiments the initial value is slightly the greater of the two. The increase in the case of the glycocoll group is very marked and amounts to 66 per cent. In the muscle the effect of the injection of glycocoll is much less pronounced and there is a comparatively slight increase of 8·8 per cent.; in 33 per cent. of the individual cases the initial value is greater than the subsequent value.

Effect of the injections on the amide nitrogen (see Tables V and XIII). The amide figures are particularly interesting; in the sodium chloride group for the liver they are practically identical before and after injection but in all the other groups there is a definite increase, which amounts to 5 per cent. after the injection of glycocoll, 16 per cent. and 17 per cent. after the injection of ammonia in the first and second group respectively, and 149 per cent. in the case of the injection of urea itself. The heaping up of urea is very striking and is obviously determined by the concentration in the blood, whereas in the case of the other groups the increase of amide nitrogen is presumably due to the conversion of some of the injected ammonia or glycocoll. In 11 per cent. of the individual experiments the amide nitrogen is diminished instead of being increased after injection. Fiske and Karsner [1913] have already been mentioned as having perfused the livers of cats and rabbits with defibrinated blood to which additions of ammonium carbonate or of glycocoll were made. They found a definite increase of urea in the blood

TABLE XV.

(*a*) *Giving the average figure for the different groups of experiments of the total soluble or non-protein nitrogen as a percentage of the total nitrogen.*

	Liver		Muscle	
Experiments	Before	After	Before	After
Early ammonia ...	9·4	9·8	10·45	10·93
Last ammonia ...	10·02	10·18	12·26	12·19
Sodium chloride ...	10·2	10·19	10·85	10·75
Glycocoll	10·14	11·61	11·10	10·84
Urea	9·32	10·2	12·11	13·47
Mean for whole series	9·5	10·3	11·1	11·2

(*b*) *Giving the average figure for the different groups of experiments of the protein nitrogen as a percentage of the total nitrogen.*

	Liver		Muscle	
Experiments	Before	After	Before	After
Early ammonia ...	90·5	90·0	89·5	89
Last ammonia ...	89·9	89·8	87·7	87·8
Sodium chloride ...	89·7	89·8	89·1	89·2
Glycocoll	89·9	88·2	88·9	89
Urea	91·6	88·8	87·8	86·5
Mean for whole series	90·5	89·7	88·9	88·8

from the ammonium salt but none in the case of glycocoll, but as has already been pointed out the conditions of the experiment were not the most favourable for urea formation.

Jansen [1915] perfused the livers of cats and dogs with ammonium carbonate and various amino acids and found an increase of urea in the blood.

In some experiments [Bostock, 1912, 2] which were carried out on the formation of amide nitrogen from glycocoll by the liver pulp in vitro no deaminisation of the glycocoll could be observed; the evidence on the whole, however, was in favour of a small amount of urea formation, for both actual and percentage amounts of amide nitrogen were increased, but the percentage was smaller than the actual increase.

The effect of injection on the ammonia (see Tables V and XIII). The total amount of ammonia in fresh tissue is very small only amounting to 2 or 3 per cent. of the total soluble nitrogen; this amount is practically unchanged after the injection of physiological salt solution but all the other groups show a definite actual and percentage increase as a result of injection. There is an increase of 19 per cent. in the early ammonia experiments, 69 per cent. in the glycocoll group, 73 per cent. in the urea and 430 per cent. in the later ammonia group. The amount of ammonia that can be injected is strictly limited by its toxicity and it would appear that some animals are more susceptible than others; the rate of injection, as well as the amount, exercises an influence on the heaping up in the tissues, the best result being obtained when the organism is flooded with the salt in question. The operation of one or other of these factors probably accounts for the greater accumulation of ammonia found in the later ammonia experiments. In 8 per cent. of the whole series of experiments the ammonia is diminished, in 8 per cent. it is unaltered and in the remaining 84 per cent. it is increased after injection.

It is very interesting to note that there has been a deaminisation of glycocoll, for Lang [1904] claimed that the same thing occurred in vitro, and some earlier experiments of my own with liver pulp seemed to indicate a slight deaminisation; the result of later work however failed to confirm this and showed the necessity of determining the soluble nitrogen as well as the ammonia in all autolysis experiments. An increase of ammonia is not necessarily derived from the amino acid added but may be due to a more rapid autolysis in the case of a particular digest or even to the presence of putrefactive organisms. In every case the percentage relationship of the ammonia to the soluble nitrogen is the important factor, and this relationship could not be determined in Lang's or in my own early work because the sole estimations were those of ammonia.

The increase of ammonia after the injection of urea is an indication of a certain amount of deaminisation. Wakeman and Dakin [1911] perfused dogs' livers with urea, but under the conditions of their experiments they were unable to detect any increase of ammonia in the blood at the expense of the urea. Janney [1911] investigated the question of whether the conversion of ammonia into urea was a reversible reaction, and after reducing the excretion of ammonia to a minimum by giving large doses of sodium citrate he administered urea but it was not followed by any increase of ammonia in the urine. Lichtwitz [1912] on the other hand criticised Janney's work adversely. He claimed to have found an increase of ammonia and of amino acid with a retention of nitrogen following on the administration of urea to one dog and to four diabetics, but his data are wholly inadequate to establish his conclusions. Marshall and Davies [1914] in their experiments on the distribution and elimination of urea found no evidence of the conversion of urea into any other body but this conclusion is apparently based on indirect evidence as there is no mention of any ammonia estimations.

The changes in the ammonia content of the muscle are much less marked than in the liver; there is an increase in the majority of individual cases, but the amount is unaltered in 18 per cent. and diminished in 9 per cent. of the experiments. In the latest ammonia experiments there is an increase of 24 per cent. but in the other groups the changes are insignificant.

When the values for the non-protein nitrogen and its constituents are calculated on the dried instead of on the fresh tissue they do not materially affect the results, as can be seen from the Tables XIII and XIV, the essential features remain the same and in some cases are even emphasised, as in the urea and glycocoll groups.

When the amino, amide and ammonia nitrogen are calculated as percentages of the non-protein nitrogen it will be seen from Table VIII that the percentage relationships reflect the changes found in the absolute values. Owing to the considerable increase in the value of the non-protein nitrogen itself after the injection of urea and glycocoll, however, the percentage increase in the amide and the amino acid nitrogen in these two groups is less marked than the absolute increase.

As Table XIII is the most important of the whole series it will be well to sum up the results it contains. The sodium chloride group is the only one in which all the values non-protein, amino, amide and ammonia nitrogen, both total and percentage, remain practically unchanged. Both the ammonia groups show a total and percentage increase in the ammonia and amide nitrogen of the liver after injection, which can only be interpreted as due to the fixation of ammonia and the conversion of some of it into urea. When the organism is flooded with ammonia, to use van Slyke's phrase, it desaturates itself by working it up into the normal product, but whether this is the only way in which desaturation takes place, the data available do not indicate. Another point of interest is that there is no indication of an amination of fatty acids occurring; possibly suitable conditions are not present, but at any rate we are justified in assuming that if amination occurs it is a much more specialised process than the synthesis of urea. Turning to the muscle in the last ammonia group the same changes are observed in a less marked degree than in the liver, but they are scarcely perceptible in the group of earlier experiments. Undoubtedly there is a fixation of ammonia by the muscle and it seems probable that the muscle possesses the power of converting this into urea without the intervention of the liver.

The glycocoll group shows a very marked fixation of amino acid by the liver and quite a marked increase in the ammonia due to a deaminisation of the glycocoll; there is also a definite but less well marked increase in the amide nitrogen due to a conversion of some of the ammonia into urea. An increase of urea in the blood after the intravenous injection or absorption of large quantities of amino acid has been abundantly shown by van Slyke and by Folin, but I am not aware of the same changes having been demonstrated in the liver, although Folin and his pupils have shown a very marked increase in the urea of the muscle under these conditions. The fixation of amino acid by the muscle is much smaller than by the liver; van Slyke has shown that it does not reach its saturation point as soon as the liver. It is to be noted that although the ammonia has not increased there is a definite increase in the amide nitrogen.

The urea group reveals changes in the ammonia and amide nitrogen, the latter is enormously increased and the former shows a very definite increase, and there can be no doubt of the deaminisation of some of the urea. The muscle also exhibits a high degree of urea fixation and a slight increase in the ammonia nitrogen.

CONCLUSIONS.

Variation is the rule in those aspects of tissue analysis which form the subject of the present communication, there is not a single instance of a constant value or relation although the range of variation is much greater in some relations than in others.

The intravenous injection of ammonia, urea and glycocoll, respectively, produces an accumulation of these bodies as such in the liver and to a less extent in the muscle. There is no evidence of the synthesis of any of these substances into protein but there is abundant proof of their further metabolism particularly in the liver; ammonia is converted into amide nitrogen in both liver and muscle, urea is deaminised in the liver and muscle while glycocoll is deaminised in the liver and is partially converted into amide nitrogen in both liver and muscle.

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XXI. THE INFLUENCE OF THE NATURE OF THE DIET ON THE RETENTION OF PROTEIN.

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The question of the capacity of the organism to store protein has always been one of considerable interest and although it is highly probable that such a storing is a normal process the critical conditions for its occurrence have not yet been fully elucidated, largely owing to the fact that the amount of information available from nitrogen equilibrium studies is small and its value difficult to assess. Still by the use of the method of superimposition results of importance can be obtained without recourse to such favouring conditions of storage as muscle work, growth or inanition, all of which conditions introduce secondary factors which render the interpretation of the data more difficult.

In the course of another investigation Tsuji [1915] found that the degree of retention of superimposed protein varied (1) with the nature of the standard diet, and (2) with the nature of the protein superimposed. As these experiments were, as regards the superimposition, only of one day's duration the present series of experiments was carried out to amplify the data obtained.

The animal employed was an Airedale bitch weighing 17·6 kilos. She was fed daily at 11 a.m. after catheterisation, the urine then obtained being added to the urine collected in the receiver of the metabolic cage.

The analytical methods employed were: total nitrogen, Kjeldahl; urea, Plimmer and Skelton's modification of the urease method; ammonia, Folin. The delimitation of the faeces was carried out by means of charcoal or carmine. The total faeces was collected for each period and a single analysis of the mixed specimens was made.

As regards the standard diets employed three different combinations of protein, fat and carbohydrate were used. They all contained the same amount of protein and were of the same caloric value but varied markedly in their content of fat and carbohydrate. They were as follows:

I. Carbohydrate-rich, fat-poor diet.

Scott's oatflour	= 120 g.		
Dried skimmed milk	= 43 g.		
Margarine	= 34 g.	Nitrogen	7·2 g.
Caseinogen	= 19 g.	Carbohydrate	194 g.
Tapioca	= 93 g.	Fat	34 g.
Sodium chloride	= 2 g.	Caloric intake	1296

II. Intermediate diet.

Scott's oatflour	= 60 g.		
Dried skimmed milk	= 62 g.		
Margarine	= 77 g.	Nitrogen	7·2 g.
Caseinogen	= 19 g.	Carbohydrate	117 g.
Tapioca	= 47 g.	Fat	68 g.
Sodium chloride	= 2 g.	Caloric intake	1296

III. Fat-rich, carbohydrate-poor diet.

Scott's oatflour	= 30 g.		
Dried skimmed milk	= 40 g.		
Margarine	= 120 g.	Nitrogen	7·2 g.
Caseinogen	= 34 g.	Carbohydrate	42 g.
		Fat	101 g.
Sodium chloride	= 2 g.	Caloric intake	1296

The standard diet was continued for a pre-period of five to eight days until the nitrogen output was approximately constant, there was then added to the diet a definite amount of protein material, and this was continued for eight days when the diet again reverted to the original standard for a post-period of six or eight days. It was thought that in this way some definite information as regards retention would be obtained.

Caseinogen experiments.

EXPERIMENT I. *Retention of caseinogen nitrogen on the carbohydrate-rich, fat-poor diet.*

In this experiment the animal was fed on the carbohydrate-rich standard diet. After a pre-period of five days 30 g. of caseinogen containing 3·6 g. nitrogen were added daily to the standard diet during eight days. Then the diet was again changed, for a post-period of seven days, to the original standard diet.

It will be noted from the Table I A that although there was a progressive fall in the retention of nitrogen from + 0·4 g. on the first day to + 0·3 g. on the fourth day and even a slight negative balance on the fifth day the average daily retention was + 0·2 g. giving a total retention of 1·44 g. for the whole period. During the chief period, I B, when caseinogen was being added to the diet there was a well-marked retention amounting to + 1·26 g. daily, with a total retention for the whole period of 10·0 g. nitrogen. As during this period 28·8 g. of nitrogen were superimposed there was therefore a retention of just over 35 %. In the post-period, I C, there was a quite definite negative balance, the average daily loss being – 0·26 g. or 1·84 g. for the total period. This loss, as it continues over the whole period, cannot in the light of Falta's [1906] careful experiments on the re-excretion of nitrogen be regarded solely as due to re-excretion. Falta found in his experiments with caseinogen that

TABLE I.

Day of experiment	Total amount of urine in cc.	Nitrogen intake in g.	Nitrogen output in g.				Daily nitrogen balance g.
			Urine			Faeces	
			Ammonia	Urea	Total nitrogen		
A. *Pre-period.*							
I	690	7·2	0·49	4·98	6·05	0·66	+0·49
II	540	7·2	0·48	5·02	6·10	0·66	+0·44
III	670	7·2	0·48	5·19	6·23	0·66	+0·31
IV	620	7·2	0·46	5·12	6·16	0·66	+0·38
V	560	7·2	0·51	5·60	6·72	0·66	–0·18
Average		7·2	0·48	5·18	6·25	0·66	+0·29
B. *Caseinogen period* (3·6 g. nitrogen in the form of caseinogen were added daily).							
VI	700	10·8	0·64	7·13	8·36	0·79	+1·65
VII	700	10·8	0·66	7·24	8·56	0·79	+1·45
VIII	580	10·8	0·67	6·63	7·91	0·79	+2·10
IX	570	10·8	0·80	7·89	9·34	0·79	+0·67
X	730	10·8	0·76	7·73	9·07	0·79	+0·94
XI	730	10·8	0·69	7·22	8·49	0·79	+1·52
XII	770	10·8	0·70	7·61	8·98	0·79	+1·03
XIII	840	10·8	0·78	7·88	9·28	0·79	+0·73
Average		10·8	0·71	7·42	8·75	0·79	+1·26
C. *After period.*							
XIV	790	7·2	0·51	6·03	7·26	0·66	–0·72
XV	900	7·2	0·49	5·83	6·93	0·66	–0·37
XVI	800	7·2	0·48	5·07	6·18	0·66	+0·38
XVII	800	7·2	0·55	5·75	6·90	0·66	–0·36
XVIII	760	7·2	0·47	5·27	6·34	0·66	+0·20
XIX	760	7·2	0·54	5·91	7·08	0·66	–0·54
XX	840	7·2	0·50	5·85	6·95	0·66	–0·41
Average		7·2	0·51	5·67	6·81	0·66	–0·26

the re-excretion ceased about the fourth day after the caseinogen was stopped. Moreover Tsuji's experiments demonstrate clearly that there is no prolonged excretion of excess nitrogen after the superimposition of caseinogen. This prolonged secondary loss is very difficult to explain except on the ground that there has been a stimulation of the total protein metabolism due to the increased intake.

EXPERIMENT II. *Retention of caseinogen nitrogen on the intermediate diet.*

TABLE II.

A. *Pre-period.*

			Nitrogen output in g.				
			Urine			Faeces	
Day of experiment	Total amount of urine in cc.	Nitrogen intake in g.	Ammonia	Urea	Total nitrogen		Daily nitrogen balance g.
I	590	7·2	0·42	5·94	6·99	0·56	−0·35
II	560	7·2	0·40	5·33	6·32	0·56	+0·32
III	530	7·2	0·46	5·65	6·74	0·56	−0·10
IV	620	7·2	0·45	5·64	6·76	0·56	−0·12
V	590	7·2	0·47	5·99	7·15	0·56	−0·51
Average		7·2	0·44	5·71	6·79	0·56	−0·15
B. *Caseinogen period* (3·6 g. nitrogen in the form of caseinogen were added daily).							
VI	620	10·8	0·68	7·68	9·07	0·65	+1·08
VII	520	10·8	0·72	7·77	9·18	0·65	+0·97
VIII	610	10·8	0·67	7·28	8·69	0·65	+1·46
IX	530	10·8	0·69	7·54	8·85	0·65	+1·30
X	670	10·8	0·69	7·66	9·00	0·65	+1·15
XI	590	10·8	0·78	8·00	9·41	0·65	+0·74
XII	610	10·8	0·73	8·16	9·54	0·65	+0·61
XIII	460	10·8	0·71	7·85	9·23	0·65	+0·92
Average		10·8	0·71	7·74	9·12	0·65	+1·03
C. *After period.*							
XIV	560	7·2	0·50	5·34	6·36	0·84	0
XV	580	7·2	0·47	5·80	6·90	0·84	−0·54
XVI	490	7·2	0·48	5·18	6·27	0·84	+0·09
XVII	610	7·2	0·47	5·04	6·07	0·84	+0·29
XVIII	540	7·2	0·43	5·11	6·09	0·84	+0·27
Average		7·2	0·47	5·29	6·34	0·84	+0·02

In this experiment the standard diet employed was the intermediate one and 30 g. of caseinogen were added during the chief period. During the after period there was a certain amount of difficulty as the faeces became semi-diarrhoeic and we may conclude that absorption from the intestine was imperfect. During the pre-period the nitrogen balance was, with the exception of one day, slightly on the negative side giving a daily average of −0·15 g., *i.e.* 0·76 g. was lost during this period. When caseinogen was added the balance was positive throughout the period giving a daily average of + 1·03 g. the total retention for the period being 8·23 g., *i.e.* 28·6 % of the nitrogen added in the form of caseinogen was retained. In the post-period there was a slight positive balance.

Experiment III. *Retention of caseinogen nitrogen on the fat-rich, carbohydrate-poor diet.*

TABLE III.

A. *Pre-period.*

			Nitrogen output in g.				
			Urine			Faeces	
Day of experiment	Total amount of urine in cc.	Nitrogen intake in g.	Ammonia	Urea	Total nitrogen		Daily nitrogen balance g.
I	360	7·2	0·45	5·44	6·56	0·73	−0·09
II	330	7·2	0·50	5·30	6·52	0·73	−0·05
III	350	7·2	0·54	5·27	6·48	0·73	−0·01
IV	400	7·2	0·56	5·33	6·54	0·73	−0·07
V	610	7·2	0·58	5·67	6·79	0·73	−0·32
Average		7·2	0·53	5·40	6·58	0·73	−0·11

B. *Caseinogen period* (3·6 g. nitrogen in the form of caseinogen were added daily).

VI	610	10·8	0·81	8·01	9·44	0·67	+0·69
VII	440	10·8	0·84	7·79	9·32	0·67	+0·81
VIII	600	10·8	0·88	8·32	9·97	0·67	+0·16
IX	750	10·8	1·03	8·49	10·21	0·67	−0·08
X	690	10·8	0·88	8·50	10·08	0·67	+0·05
XI	680	10·8	0·90	8·33	9·86	0·67	+0·27
XII	690	10·8	0·89	8·36	9·97	0·67	+0·16
XIII	660	10·8	0·87	8·13	9·74	0·67	+0·39
Average		10·8	0·89	8·24	9·82	0·67	+0·31

C. *After period.*

XIV	852	7·2	0·66	5·14	6·45	0·76	−0·01
XV	620	7·2	0·66	5·35	6·65	0·76	−0·21
XVI	640	7·2	0·63	5·38	6·65	0·76	−0·21
XVII	620	7·2	0·59	5·24	6·43	0·76	+0·01
XVIII	850	7·2	0·58	4·85	6·18	0·76	+0·26
Average		7·2	0·62	5·19	6·47	0·76	−0·03

In this experiment the dog was kept throughout on the fat-rich diet, 30 g. of caseinogen being added daily during the chief period.

During the pre-period, as in the second experiment, there was a slight negative balance which amounted to a daily loss of − 0·11 g. or of 0·54 g. nitrogen in all. In the chief period except for the first day when there was a small negative balance there was evidence of retention of nitrogen. The average balance for the eight days was + 0·31 g. and the total amount retained was 2·45 g. or 8·5 % of the total nitrogen ingested in the form of caseinogen. In the post-period during the first three days the balance was negative and on the two following days positive, giving however an average loss of − 0·03 g. nitrogen per diem.

One fact emerges very clearly from the foregoing experiments namely that the nature of the standard diet employed plays a well-defined part in the degree of retention obtained. In each experiment the same amount of nitrogen in the form of caseinogen was added to the diet but the amount retained varied in each instance. Thus in the case of the carbohydrate-rich diet there was a retention of 10·09 g. corresponding to 35 % of that superimposed; on the intermediate diet the retention amounted to 8·23 g. or 28·6 % of the superimposed amount and on the carbohydrate-poor, fat-rich diet the retention amounted only to 2·45 g., equivalent to 8·5 % of the total addition. The evidence, then, clearly points in favour of the view that carbohydrate plays some preponderant *rôle* in the retention of protein, that when a sufficiency of carbohydrate is not available there is a failure in the synthetic processes. This is borne out by the consideration, for example, of the marasmic condition associated with diabetes, where protein is given in large amount in the diet but without any gain on the part of the patient. It had been hoped that some light would have been obtained on the proportion of carbohydrate which was essential, *i.e.* on the ratio which must exist between carbohydrate and fat in a diet, but, owing to various difficulties, this point could not be fully investigated at present. From the results of Lang [1915] on acidosis it would seem probable however that the highest ratio of fat to carbohydrate which is possible is 1 : 2.

Caseinogen is generally recognised as being a form of protein which is more readily retained than many other forms [Röhmann 1898; Caspari 1900], although this has been denied by some workers [Bloch 1900] for example. As the present experiments had given such a satisfactory result the question as to whether the superimposition of an imperfect protein like gelatin would yield an equally satisfactory result arose.

Experiment IV. *Retention of gelatin nitrogen on the carbohydrate-rich, fat-poor diet.*

TABLE IV.

			Nitrogen output in g.				
Day of experiment	Total amount of urine in cc.	Nitrogen intake in g.	Urine			Faeces	Daily nitrogen balance g.
			Ammonia	Urea	Total nitrogen		
A. *Pre-period.*							
I	800	7·2	0·48	5·07	6·18	0·66	+0·38
II	800	7·2	0·55	5·75	6·90	0·66	−0·36
III	760	7·2	0·47	5·27	6·34	0·66	+0·20
IV	760	7·2	0·54	5·91	7·08	0·66	−0·54
V	840	7·2	0·50	5·85	6·95	0·66	−0·41
Average		7·2	0·51	5·57	6·69	0·66	−0·15
B. *Gelatin period* (4·1 g. nitrogen in the form of gelatin were added daily).							
VI	840	11·3	0·60	8·87	10·16	0·78	+0·36
VII	840	11·3	0·64	9·04	10·33	0·78	+0·19
VIII	600	11·3	0·60	7·86	9·09	0·78	+1·43
IX	770	11·3	0·58	8·04	9·30	0·78	+1·22
X	880	11·3	0·63	8·69	10·03	0·78	+0·49
XI	860	11·3	0·60	8·37	9·63	0·78	+0·89
XII	760	11·3	0·64	8·39	9·77	0·78	+0·75
XIII	810	11·3	0·60	7·85	9·07	0·78	+1·45
Average		11·3	0·61	8·39	9·67	0·78	+0·85
C. *Gelatin and meat extract period* (4·1 g. nitrogen in the form of gelatin and 0·5 g. nitrogen in the form of meat extract were added daily).							
I	760	11·8	0·59	8·77	10·24	0·96	+0·60
II	1020	11·8	0·68	9·55	11·08	0·96	−0·24
III	780	11·8	0·64	8·37	9·72	0·96	+1·12
Average		11·8	0·64	8·90	10·35	0·96	+0·49
D. *After period.*							
I	980	7·2	0·58	6·34	7·50	0·97	−1·27
II	1040	7·2	0·56	5·88	7·02	0·97	−0·79
III	960	7·2	0·53	5·35	6·38	0·97	−0·15
Average		7·2	0·55	5·86	6·97	0·97	−0·74

As in the previous experiments the animal was put on the standard diet, in this case the carbohydrate-rich, fat-poor diet, for a pre-period of five days, then to this diet there was added daily during the chief period 30 g. of gelatin containing 4·1 g. nitrogen.

During the pre-period the balance was very variable, the daily average for the period being − 0·15 g. In the chief period the balance throughout was positive, the average daily retention amounting to + 0·85 g., the total retention for the whole period was 6·78 g. corresponding to 20·7 % of the nitrogen taken in, *i.e.* the retention in the case of gelatin is definitely lower than in that of caseinogen under exactly the same conditions. In view of Thompson's [1910] statement that the addition of meat extract to a diet brings about a more perfect retention it was thought that the present series of experiments offered a good opportunity of testing this matter. Accordingly the chief period was continued for a further three days during which time 5 g. of meat extract were added to the gelatin and superimposed on the standard diet. As the figures in IV C show this addition produced no increase in the amount retained, indeed the daily average was definitely lowered. Unfortunately, as the animal was tiring of the diet, the post-period could not be continued for the usual length of time. (Unless every precaution is taken to see that the animals, during these tests, maintain their appetites the experiments lose a very large part of their value.) During the post-period, in the present experiment, there was a very well-marked negative balance which may be due in large part to a re-excretion of the retained nitrogen. In this respect the apparent retention is even more unfavourable than the above calculation would suggest.

EXPERIMENT V. *Retention of gelatin nitrogen on a fat-rich, carbohydrate-poor diet.*

TABLE V.

A. *Pre-period.*

			Nitrogen output in g.				
			Urine			Faeces	
Day of experiment	Total amount of urine in cc.	Nitrogen intake in g.	Ammonia	Urea	Total nitrogen		Daily nitrogen balance g.
I	750	7·2	0·60	5·47	6·70	0·53	-0·03
II	680	7·2	0·62	5·40	6·59	0·53	+0·08
III	550	7·2	0·58	4·74	5·89	0·53	+0·78
IV	630	7·2	0·62	5·50	6·79	0·53	-0·12
V	640	7·2	0·63	5·84	7·01	0·53	-0·34
Average		7·2	0·61	5·39	6·60	0·53	+0·07

B. *Gelatin period* (4·1 g. nitrogen in the form of gelatin were added daily).

I	530	11·3	0·69	8·66	10·01	0·81	+0·48
II	580	11·3	0·73	8·81	10·15	0·81	+0·34
III	560	11·3	0·62	8·94	10·21	0·81	+0·28
IV	590	11·3	1·20	8·88	9·99	0·81	+0·50
Average		11·3	0·76	8·82	10·09	0·81	+0·40

In this experiment the standard diet was fat-rich, carbohydrate-poor. The amount of gelatin superimposed was the same as in experiment IV. This experiment is not complete as after the fifth day of the chief period the animal lost its appetite and could not be induced to finish its daily allowance of food. The data recorded are all, however, free from objection.

During the pre-period again the balance was variable but in this instance it was very slightly positive, the daily average retention being + 0·07 g. In the chief period the average daily retention amounted to + 0·4 g. or 1·6 g. for the four days corresponding to 9·76 % of the total nitrogen superimposed, a result slightly more favourable than that found for caseinogen under similar conditions for a period of eight days (8·5 %), but slightly less favourable if compared with the retention of the caseinogen nitrogen for the period of the first four days (10·97 %), which is more directly comparable with the gelatin figures. Here, therefore, as in the case of the caseinogen, although the retention is not quite so marked, the carbohydrate exercises a definite retaining influence on the superimposed nitrogen.

As regards the method by which the carbohydrate acts it is not proposed to deal with the problem in the present communication. It is hoped however to take up the consideration of this question in another paper.

CONCLUSIONS.

1. Nitrogen in the form of protein added to a carbohydrate diet is retained in greater amount than when added to a fat diet of equal caloric value.
2. Nitrogen given in the form of caseinogen is more completely retained than when given in the form of gelatin.
3. The addition of meat extract to gelatin does not increase the amount of nitrogen retained.

These experiments were carried out at the suggestion and under the direction of Dr E. P. Cathcart to whom I offer my thanks.

The expenses were defrayed by a grant from the London Hospital Research Fund.

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XXII. "PLATE" FORMS OF ULTRAFILTRATION APPARATUS.

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(*Received May 4th, 1916.*)

The use of ultrafilters as accepted aids to investigation leads naturally to more practical forms of ultrafiltration apparatus than that in which observations of their properties were originally made. For particular experiments special designs will always be necessary: nevertheless, as an attempt at sound constructions for general laboratory use, which will doubtless be improved upon in the light of wider experience, I put forward those illustrated below. The larger pattern was briefly described in a previous communication in which details of a method of making suitable ultrafilters were also given [Walpole, 1915].

12″ APPARATUS—PATTERN A. DIAMETER OF FILTER 10″.

Construction of the Ultrafiltration Apparatus proper.

There are two circular metal plates 12″ diameter and $\frac{3}{8}$″ thick called the *front plate* and the *back plate* (Fig. 1). These can be bolted together by nine $\frac{3}{8}$″ bolts and three $\frac{3}{8}$″ studs and wing-nuts arranged symmetrically, the former with their centres round an $11\frac{3}{8}$″ circle, and the latter round an $11\frac{5}{16}$″ circle. Between them is a $\frac{3}{16}$″ filter plate built up of two pieces —a ring 11″ external and 10″ internal diameter, and a 10″ circular disc of exactly the same thickness over all and embossed with a pyramidal pattern on both sides. The ring just clears the bolts all round and has three small indentations cut in it enabling it to fit exactly between the three studs. These support the filter plate before the front plate is applied to it, and

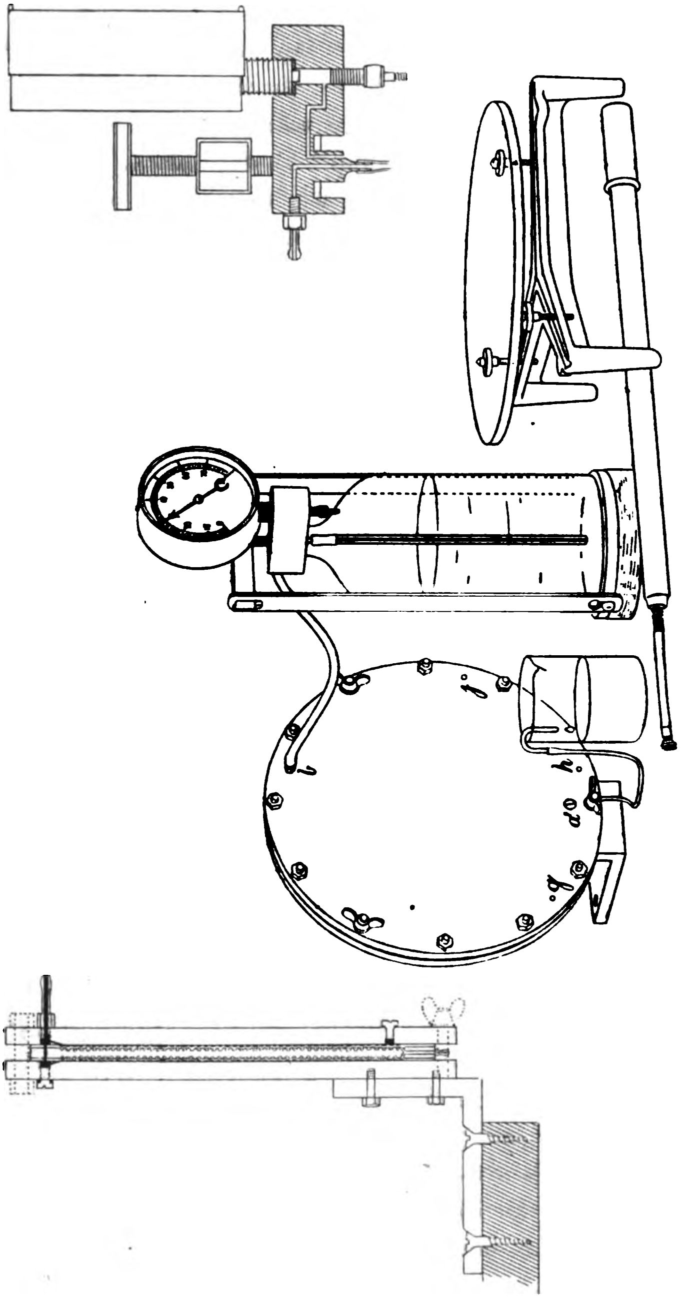

Fig. 1.

ensure that when the films are in position the front plate may be slid on gently with all the bolt holes and the holes *b*, *d*, and *a* (Fig. 2) in perfect register.

In Fig. 2 the two filters themselves are shown in position. They are indicated by the white space between two thin black lines. The metal of the three plates is indicated by cross-hatching and the fluid to be filtered entering the nipple *b* under pressure is represented by dead black. Passing through channels *x* and *y* it spreads all over both filters. The raised "pyramidal pattern" of the filter plate acts as an excellent support for the filters under pressure, and when once the filtrate (not indicated) has passed through the filter it has an unobstructed flow to the edge of the filter plate. Here it can pass through the channel *c'* out of the apparatus. A similar channel *c*, not shown, is at the top of the filter plate and connects to the outside air. The pyramidal pattern filter plate has been used by permission of Messrs S. H. Johnson, the owners of the patent.

It will be noticed that *c*, *c'* are placed in the "median line," as it were, of the apparatus, while *a*, *d*, *b*, in order that the channel they form may not cut into *c*, are placed a little to one side. For convenience of illustration the top half of Fig. 2 is a vertical section through *d*, and the lower half a vertical section through *c'*, the outlet for the filtrate.

The two holes *a*, *b* and another at *a'* are tapped No. 2 B.A. thread. Fitting interchangeably into these three tapped holes are two screwed plugs and a screwed nipple all fitted with small fibre washers.

At *g*, *h*, *j* (Fig. 1) on the front plate, and at similar positions on the back plate, another set of holes is drilled and tapped. These are seldom used, but if, at the end of a filtration, the filter plate is found to adhere to either of the other plates, separation may be immediately and safely effected by screwing brass screws into these holes.

The apparatus may be made of iron varnished and baked, or of aluminium. In the latter case rolled sheet, and not cast metal, must be used, because of the porosity of the latter. Aluminium has many advantages including that of cheapness, for there is a good deal of turning to be done, and aluminium can be cut much more quickly than iron.

To the back plate is attached a bracket, by means of which it may be screwed or clamped to the bench. As a matter of convenience the apparatus is drawn in the position shown. It is used just as easily and just as often in the inverted position which is advantageous for certain operations described later.

Thick-walled rubber tubing of capillary bore is used to lead into the apparatus through the nipple the liquid to be filtered. No wiring is required on such a joint which is perfectly sound up to quite considerable pressures (50–80 lbs. per sq. inch) and may be readily made and unmade. Capillary cycle valve tubing is used to lead off the filtrate through the small nipple at *c′*, and to admit at *c* air blowing out of *c′* the last drops of filtrate.

Method of assembling Apparatus for use.

To facilitate description it will be supposed in the first instance that a filtration is to be performed using two filters, one on each side of the filter plate. The circle of plate glass on which the collodion was poured [see Walpole, 1915, pp. 291 et seq.] is 12″ diameter so that the prepared films lying under water are all this size. One is taken, laid on a clean piece of wood or glass and, using the filter plate as template, is cut round with a knife. The trimmed film 11″ diameter is put back under water and from the cuttings a piece is quickly taken by means of scissors, taking care not to include any part from the extreme edge where oil containing collodion has been

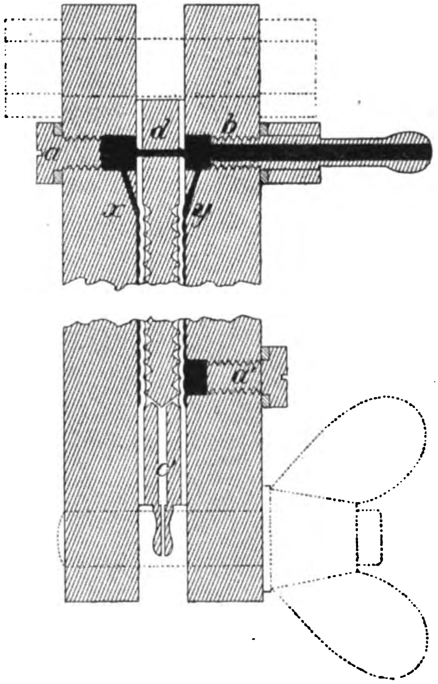

Fig. 2.

employed to prevent the film curling off the glass during drying. This piece is dried between hard filter paper without using pressure and weighed, and then dried to constant weight at 100° C. and weighed again. The ratio of these two weights gives w the wetness of the film. The value m, milligrams of dry nitro-cotton per sq. cm., is known from the volume and concentration of the collodion taken and the area of the glass plate over which it was poured.

When the two circular films each 11″ diameter are prepared, one is taken and applied carefully to the front face of the *back plate*. As it is wet it is readily manoeuvred into position and then held there by placing the *filter plate* in front of it. As has already been noticed the nipple of the *front plate* is not quite in the median line, so that in order that the continuous channel shown in the top half of Fig. 2 may be formed, care must be taken not to put the *filter plate* in back to front. The second film, thoroughly wet, is now applied to the back face of the *front plate*, the *front plate* slid on to the studs, and the whole apparatus tightened up by screwing up the wing-nuts and bolts seriatim, going round the apparatus several times. Wet films "give" considerably under pressure and the tightening up of the apparatus takes some time. The nipple on the *front plate* and the screw opposite it on the back plate are now removed and a $\frac{3}{32}$″ punch passed through so that two neat circular holes are made in the films. The screw and nipple are replaced, the rubber supply tube connected to the nipple, and filtration may be commenced forthwith. The liquid under pressure set up by the cycle pump and indicated by the gauge is forced through the passage shown in Fig. 2 over both films and the combined filtrates run away through c'. By connecting cycle valve tubing to c' the filtrate may be led to a measuring cylinder or other suitable receiver and it is certain that no small leak of the experimental liquid can pass into the filtrate.

Great trouble is experienced in summer time through flies attaching themselves to the drying collodion, so that a screen of muslin stretched on a wire frame should be provided.

A simple auxiliary device may be mentioned in passing. When say fifty cc. or less are to be handled the pressure bottle shown in Fig. 2 is obviously unsuitable. A piece of stout glass tube drawn out to a nipple at each end is clamped vertically close to the apparatus. The liquid to be forced into the apparatus may now be placed in this tube and the pressure bottle is simply used as a compressed air reservoir and is connected to the top of the tube.

Portion of the filtering area inoperative through presence of air bubbles.

The films are, of course, impervious to air and it may be thought that a certain quantity of filtering surface will not be utilised because the air in the passages will be forced on to the filter. Examination of the films, when the apparatus is taken to pieces after a filtration, shows that owing to the fineness of the passages the proportion of filtering area so rendered inoperative is very small, and may be entirely abolished if care is taken that all the channels are full of liquid before filtration commences. This being so it is a matter of indifference what the position of the apparatus is in use: in some experiments where it was desired to keep the temperature of filtration under control it was immersed horizontally in a large shallow basin: in others the bracket was screwed to the top of the table near the edge with the plates hanging down vertically.

Use of One Film only.

If one film only provides sufficient filtering area on any occasion it is convenient to put it between the *filter plate* and the *front plate*: the position of the other film between the *filter plate* and the *back plate* being occupied by a "dummy," an impervious film cut from rubber insertion. In order that the channel *da* may not be filled unnecessarily the filter plate should be put in back to front—the other channels *c* and *c′* which are symmetrically placed will of course be unaltered in position.

It is frequently desirable to recover the "ultraprecipitate," which is usually a thick gum, when the filtration is over. This may be done either by taking the apparatus to bits or by attaching a receiver to the nipple and aspirating. Washing out may be done directly by running water in and out. Except in so far as the films remain impregnated with the materials filtered off, they are recovered clean in this way, as the subsequent examination of the film plainly shows.

Although from the design of the apparatus it would appear at first sight as if the volume of fluid actually spread over the film at any one time was very small indeed, it is actually considerable, say 15 cc., and depends upon the pressure applied.

The film, of course, sags between the "pyramids" while filtration is in progress, though if the pressure is released, and still more so under suction, any remains of the liquid to be filtered are forced back through the nipple again.

Another and perhaps better way of going about the recovery of the "ultraprecipitate" is to screw in the nipple at *a′* and transfer the screw shown occupying *a′* to *b*. The liquid to be filtered is then pumped in at *a′*. The wash liquid is passed in and out at this point and *b* is used simply as an air vent.

Dialysis.

A simple procedure, which is frequently useful, for recovering a material, say a protein from a concentrated salt solution, may now be described by citing an actual example. The material, say a precipitate of crystalline egg albumin wet with ammonium sulphate solution, is to be handled with a view to obtaining the egg albumin in concentrated solution free from electrolyte. We will suppose again that only one filter is to be used. The precipitate is wetted with distilled water until fluid, and filled into a suitable glass tube drawn out at both ends (see page 258). Its lower end is attached to the nipple in *b* or *a′* and the liquid forced all over the film by air pressure from the bottle. Tap water and, later, distilled water are run across the filter plate, in at *c* and out at *c′*, care being taken to get the air out of the grooves between the pyramids first. Owing to the high osmotic pressure of the ammonium sulphate originally present some of the fluid being dialysed may regurgitate into the tube but as the dialysis and ultrafiltration proceed the ammonium sulphate is carried away. In fact, it is generally possible to regulate the pressure so that the space between the front plate and the filter is just full, *i.e.* the whole of the dialysing surface is operative. The design of the apparatus ensures extremely rapid dialysis for the volume of the liquid under treatment per sq. cm. of dialysing surface is as small as it very well can be made.

When examination of either the liquid being dialysed, or of the other liquid, or of both, shows that removal of the ammonium sulphate is sufficiently complete, then the liquid may be run off the filter plate and increased pressure applied. The egg albumin may, at the conclusion of the resultant ultrafiltration, be recovered as above.

4″ APPARATUS—PATTERN B. DIAMETER OF FILTER 3″.

For small scale operations, such as experiments on the properties of the individual members of a series of films, or to determine which film of a series is most suited for a particular operation, the simple apparatus (Fig. 3), which may be screwed up in any ordinary vice or clamp, has been found of service.

It takes one film between two pieces of aluminium, one recessed and fitted with a piece of pyramid pattern material to act as a filter plate, and the other left flat. The discs, supported and protected by being backed by pieces of iron, are suitably drilled and tapped to take the nipples, and the edges between which the film is held are ground face to face after being turned true. The perfection of this joint enables even the hardest nitro-cellulose films to be used without any leak at pressures from 50–80 lbs. per sq. inch. The films must, however, be uniform and flat.

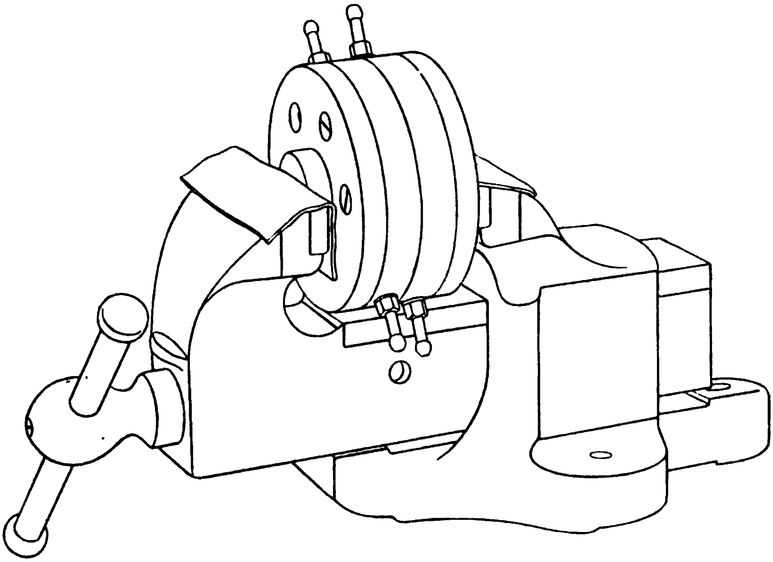

Fig. 3.

Separation of chloride from sulphate by dialysis.

I am able to confirm with flat films of nitro-cotton (British Xylonite Co.) Brown's observation [Brown, 1915] made with films of test-tube shape that this separation may be readily carried out. For assistance in obtaining suitable nitro-cotton to work on and ready advice and help in its use I am indebted and grateful to Dr J. N. Goldsmith. The following is the procedure that I have found satisfactory.

157 cc. of collodion (3·5 per cent. dry nitro-cotton in equal parts by volume of alcohol and ether) are poured on to a 12″ disc of levelled plate glass in the usual way. To prevent the drying film from curling off the glass the edge is painted round with collodion containing castor oil when half an hour has elapsed. Next day when the film is quite dry it is cut round with a penknife

using an 11½″ circular template. It is found that when perfectly dry it detaches itself readily from the plate—a uniform film which may be preserved indefinitely. Following Brown's directions this must be softened and made of the right degree of permeability by soaking in alcohol of the required strength. If a whole film is so treated the result serves admirably in the 12″ apparatus: but the small one does just as well on this occasion, so that from the original dry film four circles may be cut of ample size for this operation. Four trials may thus be made from one full-sized film. As a matter of fact twenty-four hours soaking in dilute alcohol, 50 % by volume, gives a film of the required properties. Such a film allows considerable quantities of chloride but no trace of sulphate to pass by dialysis against distilled water from a solution containing 1 % of sodium chloride and 1 % of sodium sulphate.

There have now been described two methods of preparing flat and uniform *standardised* ultrafilters of nitro-cotton and the question naturally arises as to which method is the more convenient and more reliable.

It is my opinion that where a "soft" filter is required—one, say, for separating an enzyme or a toxin from peptones, and other simpler substances—it is simpler to pour the film in the usual way and to plunge glass and all into water when drying has proceeded to the correct extent. On the other hand, where hard filters are required, and one must be prepared to differentiate, say, between those allowing dextrin to pass and those allowing the passage of sugar but not dextrin, Brown's method is certainly to be recommended.

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XXIII. ON THE PRECURSOR OF CREATINE IN CHICK MUSCLE.

By DAVID BURNS.

From the Department of Physiology, Glasgow University.

(*Received May 16th, 1916.*)

In the search for the precursor of creatine and creatinine workers naturally turned to arginine and its disintegration products. According to the theories of Knoop [1910] and Neubauer [1911], arginine is oxidised in the animal organism to guanidine-butyric acid, then to glycocyamine. Glycocyamine is then methylated to form creatine. There is no doubt that from the oxidation of arginine-containing proteins, guanidine-butyric and guanidine-acetic acids and even guanidine itself are obtained [Kutscher, 1901]. The methylation of a compound, *in vivo*, has been carried out successfully by many investigators. It is, therefore, reasonable to presume that the administration of compounds containing a guanidine or methylamino radicle will produce alterations in the creatine-creatinine metabolism.

Glycocyamine. The earliest researches were made by Czernecki [1905] who added quantities of glycocyamine to the diet of rabbits, and thereafter estimated the urinary creatinine. Using the zinc chloride method he obtained, in some instances, an increased excretion. He, however, considered that his results were indecisive.

Jaffé [1906] and his pupil Dorner [1907] observed a transformation to creatinine of from 4·5 to 12·7 % of the glycocyamine given to rabbits by the mouth. They also obtained a considerable increase in the creatine content of muscle. Dorner, too, after a study of the effect of adding glycocyamine to autolysing muscle came to the conclusion that methylation of guanidine-acetic acid to creatine takes place.

Mellanby [1908] arrived at the opposite result. He added glycocyamine in suspension to the diet of chicks on each of five days, and then determined the creatine content of their muscle and also of the muscle of the same number of controls. He found less creatine in the glycocyamine-fed chicks than in the normal.

Recently, Palladin and Wallenburger [1914, 1915] have repeated Dorner's experiments on the addition of guanidine-acetic acid to autolysing muscle. They used rabbit and eel muscle and found a decided production of creatine. In injected rabbits methylation occurred invariably, producing an increase in creatine content of from 21·5 to 35·6 %.

It is unfair to dismiss the question of the possibility of the methylation of glycocyamine or its anhydride, glycocyamidine, in the organism, on the ground that these bases do not occur naturally [Barger, 1914] and that, therefore, the formation of creatine or creatinine from them is not a physiological process. A substance may occupy a very important position in intermediary metabolism and yet be totally undetectable. It may be an absolutely necessary link in a chain of chemical changes but because of the rapidity with which it is transformed into the next substance in series, it may only momentarily exist in a free state.

Arginine. Arginine is the only known constituent of protein which contains the guanidine grouping. It has, therefore, been suggested as a possible parent of creatine and creatinine.

Negative or indecisive results were obtained by Van Hoogenhuyze and Verploegh [1905], Jaffé [1906] and Thompson [1906]. The latter worker found that 73–96 % of the arginine given was excreted as urea. More recently, however, he [1913] was able to demonstrate an increase in the creatine content of the urine after the administration of this amino-acid to dogs and birds.

Positive results are recorded by Inouye [1912], Myers and Fine [1915] and Kutscher [1914]. On the contrary, Baumann and Marker [1915] could detect no increase in total creatinine when arginine was added to hashed muscle.

Kutscher [1914] thinks that the formation of arginine is an intermediate step in the formation of creatine. It may be so rapidly transformed into the latter that detection is impossible, but in the lower organisms, for example, crustacea, the chain of chemical changes may not go so far as creatine and so arginine be found. In mammalian muscle creatine is a constant constituent and arginine unknown, while in the crayfish Kutscher demonstrated the presence of arginine and the absence of creatine.

Other possible precursors. Sarcosine may yield creatine *in vitro* [Volhard 1868, cited by Barger 1914], and Riesser [1913] administered this amino-acid in conjunction with urea, *per os* and subcutaneously, and in half of his experiments got evidence of creatine formation.

Recently [1914] he put forward the hypothesis that creatine was formed as one of the by-products of lipoid metabolism. To confirm his theory he studied the influence of injections of choline and of betaine on the creatine content of rabbit muscle, and on the creatinine elimination in urine. Choline produced positive results in four out of seven experiments while betaine gave small increases every time. Methylureido-acetic acid was perfused through isolated limbs by Baumann and Marker [1915] but without result.

Baumann, Hines and Marker [1916] perfused arginine, betaine, methylureido-acetic acid, sarcosine plus urea, and choline plus urea through muscle tissue. Only choline plus urea consistently led to an increase in muscle creatine.

It seems natural to suppose that guanidine itself might be the precursor of creatine or creatinine. It could combine with acetic acid to form glycocyamine which could add a methyl group to form creatine, or guanidine might first be methylated and then combine with acetic acid. Guanidine (or methylguanidine) may exist in muscle in two states. It may either form part of the arginine molecule or, as suggested by Jaffé and Otori, it may be the chemical nucleus to which the various peptide chains are attached.

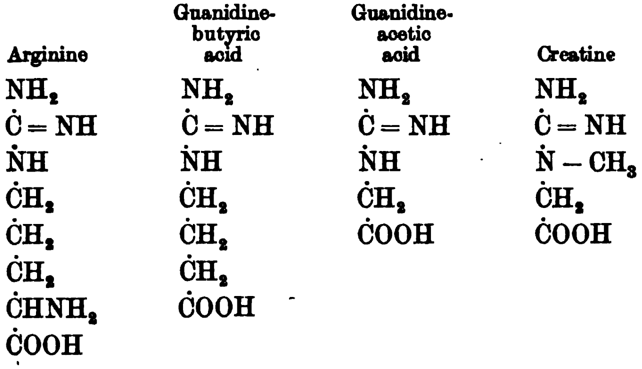
Evidence of the formation of creatine from guanidine or methylguanidine has not as yet been found. Success did not attend the efforts of Jaffé, Dorner, Pommerenig [1901] or Achelis [1906] who administered methylguanidine. Neither did Riesser [1914] who injected both guanidine and methylguanidine obtain any increase in the quantity of urinary creatine.

From a review of the literature on the subject of the origin of muscle creatine and its relationship to urinary creatinine, one must come to the conclusion that adequate facts are wanting on which to base a satisfactory theory of creatine metabolism.

Muscle of chick embryo contains no creatine up to the twelfth day of incubation. Does it contain guanidine?

In 1911, Dr Cathcart suggested to me that a study of the total guanidine content of fertilised eggs at various stages of development might throw some light on the formation of creatine. Mellanby [1908] has shown that fertilised hen's eggs contain no extractable creatine till the twelfth day of incubation, and then a trace only, which, however, increases in amount till the egg has hatched.

Kutscher [1901] suggested that possibly arginine may be the precursor of creatine. Arginine may be oxidised *in vitro* to γ-guanidine-butyric acid. Through β-oxidation this would yield guanidine-acetic acid. Jaffé, Dorner, and others have demonstrated the methylation of guanidine-acetic acid to creatine.



$$\begin{array}{llll} \text{Arginine} & \text{Guanidine-butyric acid} & \text{Guanidine-acetic acid} & \text{Creatine} \\ NH_2 & NH_2 & NH_2 & NH_2 \\ \dot{C}=NH & \dot{C}=NH & \dot{C}=NH & \dot{C}=NH \\ \dot{N}H & \dot{N}H & \dot{N}H & \dot{N}-CH_3 \\ \dot{C}H_2 & \dot{C}H_2 & \dot{C}H_2 & \dot{C}H_2 \\ \dot{C}H_2 & \dot{C}H_2 & \dot{C}OOH & \dot{C}OOH \\ \dot{C}H_2 & \dot{C}H_2 & & \\ \dot{C}HNH_2 & \dot{C}OOH & & \\ \dot{C}OOH & & & \end{array}$$

The attempts that have been made to cause a formation of creatine in the body through the administration of arginine, guanidine and methylguanidine have already been considered above.

Method.

The guanidine molecule is very resistant to oxidation. It was noted by Kutscher and his co-workers that a soluble protein, treated with successive small quantities of a boiling solution of a permanganate, yielded a complex, from which most of the products of oxidation had been driven off as volatile aldehydes, ammonia, carbon dioxide, etc. By the use of a suitable precipitant, an insoluble salt of guanidine could be obtained.

Choice of Precipitant. Guanidine is a strong monovalent base forming a series of crystalline salts with acids. Of the salts formed with mineral acids, the nitrate, $CH_5N_3 \cdot HNO_3$, is among the least soluble. It forms large plates melting at 214°.

The picrate, $CH_5N_3 \cdot C_6H_3O_7N_3$, and the picrolonate, $CH_5N_3 \cdot C_{10}H_8O_5N_4$. generally come down as amorphous precipitates, often needing repeated recrystallisation to obtain the characteristic crystals.

Guanidine picrate when pure forms irregular clusters of leaflets (Fig. 4, Plate IV). On slow heating they melt at 311–315°—on rapid heating up to 320°. Emich [1891] states that one part of the guanidine salt is soluble in 2630 parts of water at 9°. It is more soluble in hot water, slightly soluble in alcohol and very slightly soluble in ether. Dilute soda and other alkalis have apparently no effect on it while concentrated acid and alkali dissolve

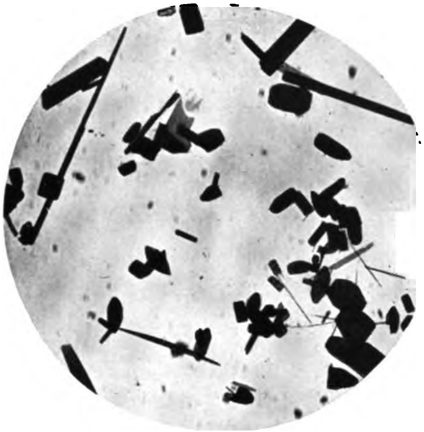


Fig. 1. Ammonium Picrate. × 50

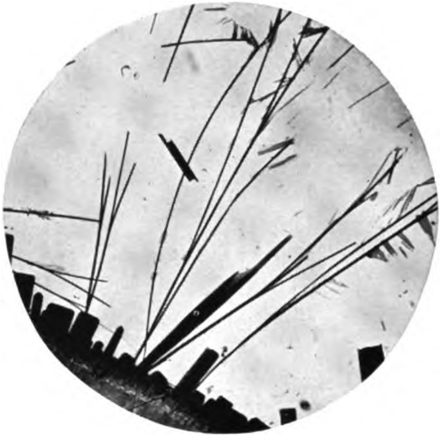


Fig. 2. Ammonium Picrate from Egg. × 50

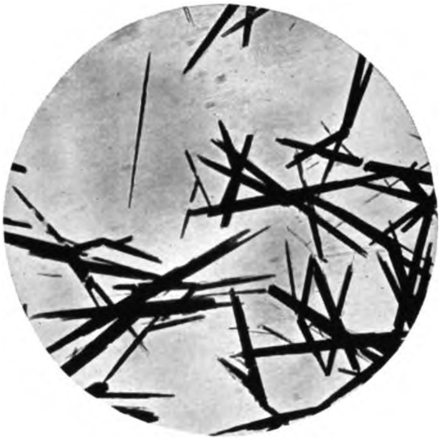


Fig. 3. Methylguanidine Picrate. × 50

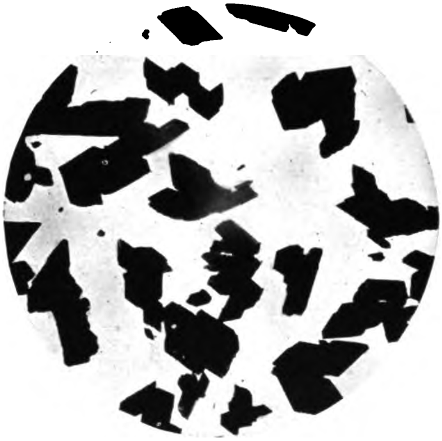


Fig. 4. Guanidine Picrate. × 50

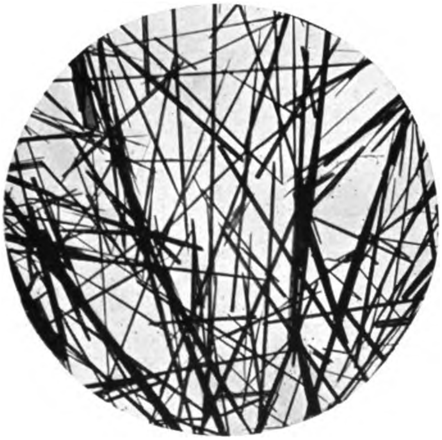


Fig. 5. Methylguanidine Picrate from Albumin. × 24

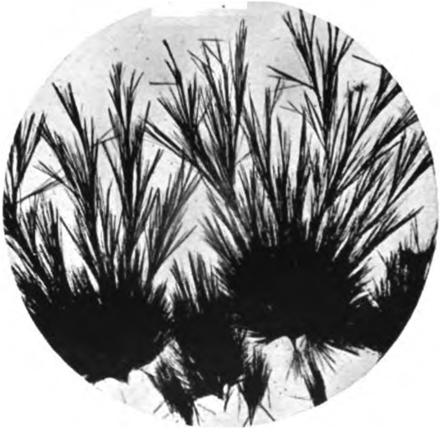


Fig. 6. Sodium Picrate. × 24

it rapidly. On heating on platinum foil it burns with a clear hissing flame leaving a coaly ash. It does not explode on heating or detonate when struck.

To precipitate guanidine from solution as picrate, the solution must not be too concentrated nor must it contain arginine [Kutscher and Otori, 1904].

Choice of Oxidising Agent. Calcium permanganate was chosen as oxidising agent, since the calcium can readily be removed as carbonate by the addition of the necessary quantity of sodium carbonate. After a trial of one or two "makes" of calcium permanganate, Merck's preparation was chosen as being most active and as being practically dry. In all the oxidations recorded below, a 10 % solution of Merck's calcium permanganate was the oxidising agent used.

Tests. Preliminary investigations were made on the guanidine content of gelatin, caseinogen, egg albumin and goose muscle.

(1) *Gelatin.*

Ten grams of commercial sheet gelatin (which gave a slight but distinct xanthoproteic reaction) were dissolved in 250 cc. water, and placed in a long-necked, round-bottomed Jena flask of one litre capacity, and the solution was brought to boiling point over an open flame. The solution of calcium permanganate was added, drop by drop, to this boiling fluid. The first drops produced a violent effervescence, and they were rapidly decolorised. As more permanganate solution was added, the effervescence became less marked, and the surface tension of the boiling fluid became greater, producing large bubbles. The boiling point of the semi-oxidised fluid was also raised. Finally, the surface tension returned to about its normal value, but each drop of the oxidising agent took a much longer time to become reduced.

The process of oxidation was stopped, either (*a*) when a certain quantity of calcium permanganate had been added, namely, 50 g.: or (*b*) when the last drop of permanganate was reduced only after prolonged boiling.

It will be seen that the oxidation process is divided into three periods.

(1) Early stage. This part is easily carried out and requires only occasional attention, for the purpose of regulating the rate of dropping of permanganate. There is a steady evolution of the aldehydes of the higher fatty acids, ammonia and carbon dioxide. Hydrocyanic acid was detected, and its presence confirmed by chemical means.

(2) Intermediate stage. Constant attention was necessary at this stage. Oxidative by-products were formed which raised the surface tension of the

fluid, and these had to be boiled off before another drop of the oxidising medium could be added. There was less smell of aldehyde and more of ammonia among the volatile by-products.

(3) Final stage. This stage presented no great difficulty. Its inception was sudden.

After cooling, the black manganese precipitate was filtered off on a Buchner funnel. The precipitate was transferred to the oxidising flask and lixiviated with about 250–300 cc. of hot water for six to eight hours, and was then cooled and filtered as before. This was repeated a second time. The filtrate and washings were united, evaporated on the steam bath to about 30–50 cc. and allowed to stand for twenty-four hours. The precipitate formed was then filtered off and washed with a small quantity of cold water. The filtrate was completely freed from calcium by the careful addition of a saturated solution of sodium carbonate, excess being avoided. The mixture was gently warmed, allowed to stand for about an hour, and then the calcium carbonate was filtered off. The filtrate which should be about 100 cc. was made up to that volume (approximately), and treated with a saturated solution of pure recrystallised sodium picrate. The guanidine picrate was allowed to stand in the mother liquor for twenty-four hours, filtered off, washed with a small quantity of cold water and then with ether. It was dried at 60° and weighed.

Results.

(*a*) Complete oxidation of 10 g. gelatin ...	1.	0·777 grams picrate
	2.	0·772 ,, ,,
	3.	0·773 ,, ,,
(*b*) Oxidation with 50 g. $Ca(MnO_4)_2$ ...	4.	0·401 ,, ,,

Two estimations of nitrogen were made of the picrate obtained from gelatin (2) and (3) (combustion process).

N of (2) 29·3; of (3) 30 %.

The picrate from gelatin (4) yielded 29·09 % of N.

Guanidine picrate contains 29·16 % nitrogen.

The substance did not melt below 280°. It was identified microscopically as guanidine picrate. (See later under "eggs.")

From the work of Kossel and Kutscher, we know that pure gelatin contains 9 % of arginine. Kutscher and Zickgraf [1903] consider that 10 g. of commercial gelatin correspond to 8 g. of the absolutely dry pure substance. Theoretically, 8 g. of pure gelatin should yield 1·19 g. of guanidine picrate.

The above workers actually got 0·75 g. from 10 g. of commercial sheet gelatin, that is 63 % of the theoretical yield. Our figures are sufficient to show that, under standard conditions, the method used is a comparative one for gelatin.

(2) *Application of the method to substances which are insoluble in water.*

Two methods are available for getting the insoluble protein material into solution, viz.:

I. Digestion with a proteoclastic enzyme.

II. Hydrolysis with a hot mineral acid.

Tests were carried out on commercial gelatin by both methods.

I. (*a*) 10 g. of gelatin were digested with 250 cc. of a 0·4 % solution of sulphuric acid, to which a small quantity of pepsin was added. The digestion lasted five days at 40°.

I. (*b*) The same quantity of gelatin was digested for the same time with 250 cc. of a 0·2 % solution of sodium carbonate, to which some pancreatin had been added.

II. The same quantity of gelatin was hydrolysed for nineteen hours with 250 cc. of 25 % sulphuric acid.

I (*a*) and II were then almost neutralised by the addition of less than the calculated quantity of barium carbonate, and were then oxidised as above, without the removal of the barium sulphate. I (*b*) was oxidised as it was.

Results.

A. *Complete oxidation.*

Peptic digest ...	0·6348 g.	guanidine picrate
Tryptic digest ...	0·23 ,,	,,
Acid hydrolysis	0·834 ,,	,,

B. *Oxidised with* 50 *g. permanganate.*

Peptic digest ...	0·4446 g.	guanidine picrate
Acid hydrolysis	0·4459 ,,	,,

It will be seen that oxidation after acid hydrolysis is productive of a greater yield of guanidine than after the substance has been brought into simple solution with water, or by means of a peptic digest. This is in accordance with the results of Kunze [1910] and Henzerling [1910] who found that an alkylated creatinine gave (on oxidation) a better yield of a substituted guanidine if it had previously been hydrolysed with acid than if it had been digested with pepsin or trypsin or both. Apparently, the enzymic splitting of the molecule differs from that produced by acid hydrolysis.

(3) *Caseinogen.*

Several attempts were made to isolate guanidine from the oxidation products of commercial caseinogen.

Two experiments using a peptic digest, and two using a pancreatin solution, were made. In all cases a paranuclein was formed. Twice, caseinogen (10 g.) was hydrolysed for twenty hours with sulphuric acid and then, after being almost neutralised, was oxidised with calcium permanganate. In no case was any guanidine found.

30 g. of caseinogen were shaken up with 300 cc. of a dilute acetic acid solution and then oxidised direct. No guanidine was isolated.

According to Hart [1901] pure caseinogen contains 4·8 % of arginine and therefore should yield about 1 % guanidine. The commercial commodity probably contains less. It follows that the method detailed above is not suitable for the isolation of small quantities of guanidine from large amounts of material.

(4) *Muscle.*

An attempt was made to determine the guanidine content of goose muscle.

I. 10 g. of minced goose muscle (moist) were digested for seven days at 40° with pepsin and hydrochloric acid, as above.

II. The same quantity of goose muscle was similarly treated with pancreatin solution.

They were both completely oxidised, as above. The peptic digest required almost 20 g. and the pancreatic digest 16 g. of permanganate, for complete oxidation.

Results.

I. Peptic digest of goose muscle gave 1·72 g. of guanidine picrate
II. Tryptic „ „ „ 1·94[1] „ „

That is, moist goose muscle yields from 0·34–0·39 % guanidine.

Nitrogen in the picrate by combustion. I = 29·1 %. II = 24·7 %.

Dr Cathcart took samples of the above muscle, and, by the method described by Brown and Cathcart [1909], estimated the creatine content.

I. Leg muscle, 0·387 % creatine
II. „ 0·393 „

Creatine contains 45·7 % guanidine. That is 0·17 and 0·18 respectively should be subtracted from the guanidine percentage given for muscle in order to obtain a figure for the non-creatine guanidine. Muscle, therefore, contains some other source of guanidine than creatine as it yields from 0·17–0·2 % non-creatine guanidine.

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[1] This was almost entirely methylguanidine picrate contaminated with a small amount of sodium picrate.

(5) *Deaminised protein.*

In the process of auto-deaminisation albumins are altered beyond the stage of amino-acids. Kutscher [1901] failed to obtain arginine from auto-digested thymus.

Reaction with either nitrous acid, or with an alkali nitrite and acetic acid, is supposed to destroy completely the $CO \cdot NH_2$-group of the protein while it does not alter the protein in such a way as to prevent the biuret reaction. It has been shown that a deaminised protein may be hydrolysed into products containing NH_2-groups.

In order to ascertain whether deaminised gelatin or caseinogen contained guanidine, 50 g. commercial gelatin were thoroughly shaken on an electric shaker with 500 cc. of 10 % acetic acid. The resultant mixture was transferred to a large basin, and was warmed to less than 40° on a water bath. A 10 % solution of sodium nitrite was added drop by drop to the well stirred liquid, till a drop no longer produced foaming. The precipitate was filtered off, washed with water and then with alcohol, and finally with ether, and dried *in vacuo.*

10 g. at a time of this deaminised gelatin were hydrolysed, and completely oxidised, as above. No guanidine picrate was obtained (two expts.). In the same way, deaminised caseinogen was prepared and oxidised. Guanidine was not obtained.

Therefore, from the gelatin experiments, one may conclude that nitrous acid destroys the guanidine radicle and also the arginine molecule. Gergens and Baumann [1876] and Pommerenig [1901] demonstrated that both guanidine and methylguanidine underwent little or no deaminisation in the animal body. It is, therefore, probable that guanidine, as such, does not exist in muscle, except perhaps in the merest traces.

(6) *Pure Egg Albumin.*

4 g. of Merck's Egg Albumin (powder) were hydrolysed and oxidised as above.

Results.

(1)	0·254 g. of the picrate.	(Nitrogen	28·7 %.)
(2)	0·288 „ „	(„	27·8 %.)
(3)	0·238 „ „	(„	27·9 %.)

Microscopic examination showed that the bulk of the yield was methylguanidine picrate. No. (2) contained a very small quantity of sodium picrate.

Guanidine hydrochloride ($CH_5N_3HCl \cdot H_2O$) should yield 253 % of the picrate.

0·2 g. of the hydrochloride was boiled up with acid and permanganate as above. 0·512 g. of the picrate was obtained (nitrogen 27·5 %); this contained traces of the precipitant.

0·2 g. of guanidine hydrochloride + 4 g. of egg albumin powder were hydrolysed, neutralised and oxidised as before. Yield, 0·875 g. of the mixed picrates of guanidine and methylguanidine.

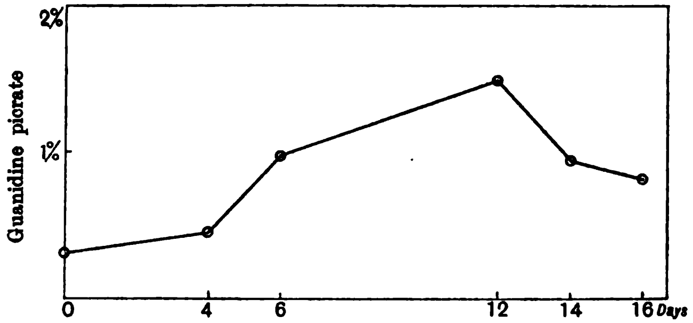
(7) *Eggs.*

The fertilised eggs, on arrival, were carefully repacked, and allowed to stand for twenty-four hours. They were then placed in the laboratory incubator kept at 40°, the air being saturated with moisture at that temperature. Each day, at the same hour, the eggs were turned by hand. At certain periods, indicated below, two eggs (Set I only) were removed from the incubator and their weight and the weight of their shell taken. The embryo was carefully examined as to development, and if its age, thus determined, did not agree with that calculated from the number of days spent in the incubator, it was at once rejected. (I am much indebted to Dr W. Abel—Mrs Shaw Dunn—for her kind help in adjudicating ages.) The embryo was then transferred to the oxidising flask and roughly extracted with ether. This removed most of the fatty matter of the yolk (triolein and tristearin), the colouring oil (chromatin) as well as cholesterol and ovine. The resultant matter was hydrolysed with 250 cc. of 25 % sulphuric acid for twenty hours, almost neutralised by the addition of 140 g. of barium carbonate, and oxidised with 50 g. of calcium permanganate, as above. That the results of this tedious process are at least comparative is shown by the similarity of the figure obtained for eggs of the same age. For example, the amount of guanidine in an egg incubated for fourteen days is given (Set I) as 0·47 and 0·44 g. of the picrate. If this is calculated as the amount of the base per 100 g. of egg substance, the figures are even closer, viz.: 0·19 % and 0·188 %. For eggs which had been incubated for about two and a half days, one gets (Set II) 0·24 % and 0·236 % of guanidine. Eggs of different sets are also comparable age for age. From Sets II, III and IV, results may be obtained for eggs of three days' incubation, namely, 0·298 %, 0·3 % and 0·3 % guanidine. The results are tabulated below, and are also presented graphically, Set I in Graph 1, and the others in Graph 2. The first series is not strictly comparable with the later sets, as the process was slightly improved in detail.

It was found to be useless to carry out estimations of the N content of the picrates as the yields consisted of mixtures of guanidine and methylguanidine. Microscopic analyses had to be taken as the sole criteria of composition, and photomicrographs of various substances met with are given in Plate IV.

Results. *Set I. May,* 1911.

Days incubated	Weight of egg g.	Weight of shell g.	Weight of material taken g.	Guanidine picrate g.	% Guanidine picrate	% Guanidine (base)
0	68·7	8·3	60·4	0·1828	0·30	0·06
4	56·6	5·84	50·76	0·231	0·45	0·09
6 +	60·9	6·9	54	0·5328	0·98	0·196
12	60·45	6·75	53·7	0·779	1·45	0·29
14	56·9	7·7	49·2	0·4707	0·95	0·19
14	54·35	7·15	47·2	0·44	0·94	0·188
16	59·52	7·75	51·77	0·4295	0·82	0·164



Graph 1. Eggs—Set I

In Sets II, III and IV slight alterations were made in the method of analysis. For instance, the barium sulphate, formed on neutralising the products of acid hydrolysis, was filtered off, and was extracted three times by boiling with water. Further, oxidation was carried out more slowly. Care was taken that as much as possible of the volatile products of oxidation was driven off by boiling before any additional permanganate was dropped in. The same quantity of permanganate was used—50 g. The final liquid was reduced further in bulk, before the precipitation of guanidine by sodium picrate.

Set II. Incubation started March 11, 1912.

Date	Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
20/3/12	48 hrs.	56·6	6·3	50·3	0·6126	1·2	0·24
23/3/12	56 hrs.	59·0	6·95	52·05	0·636	1·2	0·24
,,	,,	57·4	7·47	49·93	0·5835	1·18	0·236
22/3/12	3 days	57·25	6·05	51·2	0·7656	1·40	0·28
,,	,,	55·45	6·62	48·83	0·7759	1·5	0·3
21/3/12	4 days	56·27	6·7	49·57	0·9005	1·82	0·364

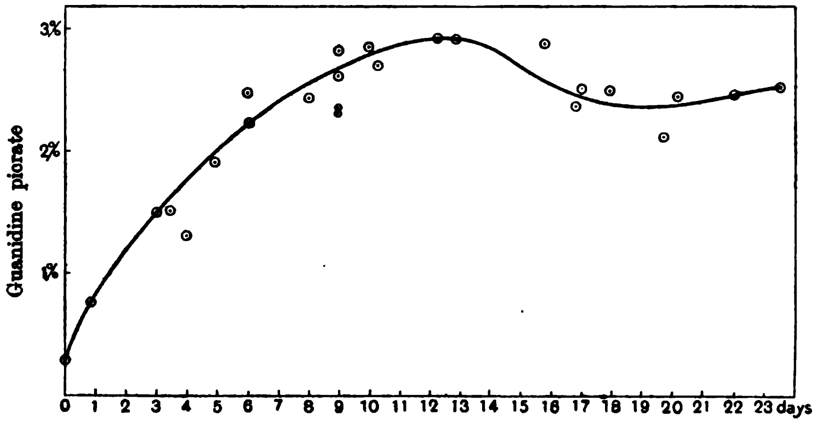
Set III. Incubation started May 31, 1912.

Date	Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
June 3	16 hrs.	60·05	5.9	54·15	0·4315	0·79	0·16
„ 4	3 days	54·95	5·85	49·1	0·76	1·5	0·3
„ 5	4 „	52·3	6·25	46·05	0·586	1·3	0·26
„ 7	6 „	56·09	6·59	50·05	1·2727	2·5	0·5
„ 10	9 „	53·61	6·4	47·21	1·3593	2·8	0·56
„ 10[1]	8 „	47·69	5·96	41·73	1·3076	3·1	0·62

[1] June 10, 1912. Age 8, had died the day before removal. Guanidine picrate contaminated with larger reddish crystals of ammonium picrate (Fig. 2, Plate IV).

Set IV. Incubation started June 25, 1912.

Date	Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
July 4	9 days	53·3	6	47·3	1·1603	2·6	0·52
„ 5	3 or 4 days (dead)	53·6	6	47·6	0·6951	1·5	0·3



Graph. 2. Sets II, III, IV and V

The incubation of the eggs in the laboratory incubation chamber had to be abandoned as it was difficult to keep the eggs alive more than nine days.

The following was the death rate in the earlier sets.

Set I.	6	died before the ninth day.
„ II.	8	„ „ „
„ III.	7	„ „ „
„ IV.	11	„ „ „

That is, over 66 % of the eggs had to be rejected as undeveloped.

Arrangements were then made with Mr A. B. Templeton, the proprietor of the "Sussex Hatcheries," to forward eggs to the laboratory as required. The eggs were removed from his incubator at 8.30 a.m., placed in a receptacle designed to retain warmth, and sent by special messenger to the laboratory where they were at once weighed, examined and submitted to acid hydrolysis.

In this series the removal of ether-soluble bodies was omitted to save time. The actual result, however, of the omission was to cause additional trouble. The presence of oily matter should, of course, have kept the surface tension of the fluid low. The oxidation products of fats seem to have the opposite effect. The second stage of the oxidation process was marked by a particularly high surface tension which produced intense frothing. This necessitated careful heating often over several days and so the process was considerably lengthened. The volatile products of oxidation were particularly odorous. In addition to hydrocyanic acid and the other bodies already mentioned, butyric aldehyde and acid were noticed.

Set V. Incubated at Sussex Hatcheries.

Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
5 days	58·8	5·915	52·885	1·0128	1·91	0·38
8 „	52·6	4·85	47·75	1·1666	2·44	0·49
9 „	53·3	5·9	47·4	1·1062	2·33	0·466
9 „	49·7	6·0	43·7	1·0123	2·3	0·46
10 „	52·67	6·5	46·17	1·3068	2·83	0·566
10 „	51·5	6·3	45·2	1·1881	2·70	0·54
11 „	53·03	6·01	47·02	1·1744	2·50 (error)	0·50
12 „	49·92	6·32	43·6	1·2956	2·94	0·59
13 „	49·95	5·6	44·35	1·3013	2·93	0·586
14 „	56·1	6·4	49·7	0·9975	2·01[1]	0·42
15 „	55·22	5·99	49·23	1·3297	2·70	0·54
16 „	53·25	5·78	47·47	1·3756	2·89	0·58
16 „	45·5	5·25	40·25	0·7832	1·94[1]	0·39
17 „	50·95	5·99	44·96	1·0692	2·37	0·474
18 „	52·65	6·9	45·75	1·166	2·5	0·5
19·75 „	52·0	6·65	45·35	0·961	2·11	0·42
20 „	49·6	5·7	43·9	1·0691	2·43	0·486
22 „	—	—	39·1	0·9754	2·49	0·498
23·5 „	—	—	34·8	0·8886	2·55	0·51

[1] Accidentally allowed to evaporate to dryness after filtration from Mn. ppt.

Great differences were noted in the difficulty of oxidising different eggs. Those at both ends of the series were fairly easy to work with—*i.e.* up to eight days and after sixteen days—while it was with great difficulty that the eggs in the middle of the series were oxidised.

Egg No. 18—age ten days—was particularly refractory. About half way through the oxidation, that is when about 25 g. of permanganate had been added, a very high surface tension was developed. The last 10 g. of permanganate were added with great difficulty. Each drop caused a persistent frothing which subsided after prolonged boiling. At times, the oxidising agent was being added at the rate of one drop in thirty minutes.

Before adding sodium picrate, precautions were taken to remove any ammonia present in solution. After the removal of the guanidine picrate formed, the mother liquor was evaporated down and allowed to stand. In most cases, a crop of crystals of the picrates of the mixed methylguanidines, was obtained. On further reduction of volume, ammonium picrate crystallised out in small amount.

The results of Sets II to V are plotted in Graph 2.

Discussion of Results.

The seven eggs in Set I show an increase in total guanidine content from 0·06 % of the unincubated egg to 0·29 % of the twelfth day of incubation. Thereafter, the percentage amount decreases rapidly to the fourteenth day and more slowly to the sixteenth.

The thirty-one eggs forming the other series represent ages from a one day egg to a two and a half day old chick (unfed). The results are plotted on the second graph, and it will be seen that both curves reach their maximum at the same point, namely the twelfth day. There is, as before, a steady increase in the amount of guanidine present from 0·16 % to 0·59 %. The decline after the twelfth day is not as regular as the incline before it. Just before the maximum is reached, there is a slight oscillation in value. On the ninth day, for instance, a slight dip occurs on the curve. If the curve were to remain smooth, there should be 0·56 % guanidine in the egg on that day. The actual figures are 0·5 and 0·46 %. The eleventh day embryo contains much less guanidine than one would expect from a study of the content of the tenth and twelfth day embryos. The filtrate from this guanidine precipitate contained a larger quantity of ammonium picrate than usual. Similarly, the 14 and 16·5 day old eggs are poorer in guanidine than one would naturally expect.

The first question that arises is, what is the cause of the twelfth day maximum?

(1) Mellanby [1908] has shown that, at that stage of development, creatine makes its first appearance as an extractable constituent of egg bioplasm. One might, therefore, consider that guanidine or its methyl derivative is continuously formed till its concentration is about 0·3 % of the

total weight of the egg. At that point, a rupture of the guanidine-containing molecule takes place with the formation of methylguanidine-acetic acid. This formation of creatine, however, does not seem to me to account for the subsequent decrease in the total guanidine content of the egg. The presence of creatine, if synthetic, might increase but in no way could decrease the amount of guanidine. It has been demonstrated that both creatine and its anhydride are readily oxidised, by the process used above, to guanidine and methylguanidine.

(2) Neither can it be that an equilibrium has been established between guanidine and creatine, for then after the twelfth day till hatching, the total guanidine would remain constant.

(3) This would also be the result if the complex containing guanidine ceased to be formed.

Two other possible explanations present themselves.

(4) The configuration of the protein complex may have been altered so that the new complex is more resistant to oxidation.

(5) It has been shown above that enzymes may split the protein complex through the guanidine molecule, so that the product gives a lower yield of guanidine on subsequent oxidation (page 269). From the work detailed above (page 271) on the action of nitrous acid on the guanidine content of gelatin, one concludes that the guanidine group (in arginine) remains free in the linking together of amino-acids to form the protein complex. This is shown in the diagram of the protein molecule given by Plimmer [1913, p. 10]. The position of arginine renders it open to the attack of enzymes, especially trypsin. Something of this nature may take place with the result that the precursor of guanidine which has not been converted into creatine is partially destroyed.

I fail to understand Mellanby's reasoning when he correlates the amount of creatine present in chick embryos of different ages with the development of the liver. There seems to be no striking alteration in either the weight or the activity of this organ on the twelfth day of incubation.

Further Noël Paton has clearly demonstrated the absence of any direct connection between the liver and creatine metabolism in the bird.

Important changes preparatory to hatching take place on the seventeenth to the nineteenth days. This is shown embryologically by the total absorption of the amniotic fluid, the retraction of the intestine and of the yolk-sac into the body cavity and the position of the head. Mellanby's curves—body weight, liver, and creatine—all show a flattening at this stage indicating an arrest in development during the period of rearrangement. The amount of guanidine also varies irregularly during those days. There is primarily a rise followed by a slight drop. On the twentieth day a steady increase begins, lasting, at least, until two and a half days after hatching.

What importance may be attached to this arrested development and oscillation of the total guanidine content just before hatching can not yet be discussed.

Very little can be said at present of the relative proportions of guanidine and the various methylguanidines obtained from developing eggs. The ease with which methylation takes place has already been alluded to, and one cannot but fear that much of the methyl derivative obtained is artefact.

Before any very decisive opinion could be expressed on the relation of guanidine to creatine formation in the developing egg, further work is necessary. When opportunity offers, a study of the free (extractable) guanidine as apart from that combined in protein or in creatine is to be carried out.

SUMMARY.

1. In the developing chick embryo there is a regular increase of the guanidine content till the twelfth day of incubation.

2. Thereafter there is a marked decrease, followed by a period of oscillation and finally a slight but steady rise.

This research was begun under the tenure of a Carnegie Scholarship, and continued by the aid of an annual grant from the Carnegie Trustees to whom I am much indebted. I am also under a debt of gratitude to Professors Noël Paton and E. P. Cathcart for their helpful guidançe and kindly criticism.

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XXIV. ON THE CONCENTRATION OF ANTITOXIC SERA.

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Of the various methods which have from time to time been proposed for the concentration of sera, those developed by Banzhaf have been most widely adopted on a commercial scale. In this communication it is only necessary to refer to:

A. *The Banzhaf-Gibson* [1907] *Two Fraction Method,* in which serum or plasma, diluted with one-third its volume of water, was heated to a temperature of 57° for 15 hours, or of 58° for 7 hours. The liquid was then made 30 per cent. of saturation with ammonium sulphate[1] and the ensuing precipitate (Fraction I) separated by filtration.

The Fraction I precipitate was suspended in saturated brine solution for 48 hours or longer. The liquid was filtered from the insoluble euglobulin and the filtrate, containing in solution the pseudoglobulin and antitoxin which had been present in the Fraction I precipitate, was made 0·25 per cent. with glacial acetic acid. The ensuing precipitate was filtered, pressed and dialysed.

The filtrate from Fraction I was made 54 per cent. of saturation with ammonium sulphate and the precipitate (Fraction II) thus formed was filtered, pressed and dialysed.

The dialysis residues from Fractions I and II were mixed, the requisite amount of salt and preservative added, and finally filtered ready for distribution. This method has been used in these laboratories for some considerable time and it has been found that by this process a good end product can be obtained which remains clear for years. Care must, however, be taken in the first precipitation, as otherwise some of the insoluble globulin passes on and appears in the final product which, in this case, does not remain clear.

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[1] Throughout this paper the phrase "made x per cent of saturation with ammonium sulphate" has been used to indicate the following: To $100-x$ parts of the serum by volume there were added x parts by volume of a saturated aqueous solution of ammonium sulphate.

B. *Banzhaf's* [1913] *One Fraction Method,* in which serum or plasma, diluted with half its volume of water and made 30 per cent. of saturation with ammonium sulphate, is brought up to a temperature of 60°, the entire heating process taking about two hours.

According to Banzhaf the filtration of the hot serum mixture should proceed rapidly. The precipitate of euglobulin is pressed well or is washed with 33 per cent. saturated ammonium sulphate; the washings are filtered and added to the main bulk of the filtrate which is then made 50 per cent. of saturation with ammonium sulphate. The ensuing precipitate, consisting of pseudoglobulin and antitoxin, is filtered, pressed, dialysed, and the dialysis residues treated in the same way as in the Banzhaf-Gibson process.

Banzhaf considers that, during the shortened heating of the mixture in the new method, a certain amount of pseudoglobulin is converted into euglobulin, and bases this conclusion on his own experiments [1908]. The effect of heating the serum mixtures is thus to ensure the precipitation of an amount of euglobulin which ordinarily would require a higher percentage of ammonium sulphate for precipitation, and which does not, as would happen in the latter case, carry down with it an appreciable amount of pseudoglobulin and antitoxin.

Banzhaf claims that the new process possesses the following advantages over the former Banzhaf-Gibson process.

(1) There is only one finished product for dialysis.

(2) There is a considerable saving of time for the reason that the first precipitate (30 per cent. of saturation with ammonium sulphate), instead of being subjected to the more lengthy process of extraction with saturated brine and subsequent precipitation of the brine extract with acetic acid, is washed with 33 per cent. ammonium sulphate and the washings added to the main filtrate.

(3) The expenses incurred in the process are considerably less, both as regards labour and the use of precipitating materials, etc.

This new method certainly possesses the advantage of being a much shorter process than the former Banzhaf-Gibson method, but unfortunately it is not always easy to work. The difficulty that sometimes arises in carrying out the process is that the serum will not filter well. This difficulty has arisen in the course of my work both in an English and in a Canadian laboratory; in the latter laboratory I had the advantage of consulting Dr Banzhaf personally about the matter. He suggested that a slight alteration in temperature or in the concentration of ammonium sulphate might be tried to overcome the difficulty, but these suggestions, although of value in some cases, did not always provide a satisfactory solution to the problem. The majority of workers on the subject with whom I have discussed the question have been faced with the same irregularity in concentrations by Banzhaf's new method.

Practical experience shows that the production of a readily filterable end product which will remain almost permanently clear, goes hand in hand with the rapid filtration of the serum mixtures after the preliminary heating process. When the heated mixtures filter with difficulty it is in some cases necessary to allow the dialysed pseudoglobulin-antitoxin precipitate to stand in the ice chest for weeks in order that the opalescent suspension therein may settle—otherwise filtration through filter candles is accomplished with great difficulty and, moreover, the final product will not remain clear.

This delay is a great disadvantage, and is one which nullifies any gain in time which should accrue from the fact that there is only one fraction to be considered.

It was obvious that the technique of the new process called for improvement or for enlargement, and in this laboratory we have ascertained that the above mentioned irregularities in the filtration can be obviated by the addition of sodium chloride to the serum mixtures, as described below.

The serum or plasma is diluted with one-half its volume of water, made 30 or 31 per cent. of saturation with ammonium sulphate and to the mixture is added 1·5 per cent. of solid sodium chloride. The temperature of the mixture is gradually raised to 61°, 63° or even 65° and kept at this point for a few minutes only. The mixture is allowed to cool to 40°–45° and filtered.

In every case the filtration of this hot mixture and of the subsequent 50 per cent. saturation proceeds rapidly. The liquid obtained after dialysis of the pseudoglobulin-antitoxin precipitate shows no sign of the presence of an opalescent colloidal suspension. After filtration through muslin it is as clear to the naked eye as though it had already been passed through paper pulp. On standing in the ice box these dialysis residues remain clear as there is nothing in opalescent suspension to be deposited.

On the addition of cresylic acid or tricresol to the dialysis residues a certain amount of precipitate is formed in all cases. In the batches of concentrated serum to which the 1·5 per cent. of solid sodium chloride has been added this deposit is exceedingly slight, even after the serum has stood many weeks. Such a serum may undergo its final filtration through pulp and filter candles almost at once: the end product will remain perfectly clear.

By the addition of 1·5 per cent. solid sodium chloride to the serum mixtures we have so far obtained a satisfactory end product with certainty and the loss of antitoxic units has been very small; a most important item to be considered in assessing the value of any method. From incomplete experiments we have reason to believe that by a further study of the dilution of the plasma, of the concentration of the ammonium sulphate and of sodium chloride and of the general treatment of the mixture, we may be able to reduce the loss of antitoxin to a minimum and at the same time produce a much better end product.

At Dr MacConkey's suggestion, an effort has been made to control the heating of the serum by means of the immersion refractometer, in order to see if some easy indication might be obtained as to the best point at which to interrupt the process. This appeared hopeful in view of Banzhaf's explanation of the effect of heating. However, the results obtained from the use of this instrument, of which the reliability and accuracy have been well established, have demonstrated that no useful indication was forthcoming, for, contrary to Banzhaf's hypothesis, during the heating process there is no appreciable change in the amount of protein in solution in the serum mixture. This result, not entirely unsuspected, has led to some interesting investigations which have been carried out with routine and with experimental batches of serum in order to elucidate:

I. The value of the heating process in the new One Fraction Method.

II. The part played by the sodium chloride in improving the technique of Banzhaf's new process.

I. *The value of the heating process in the Banzhaf new One Fraction Method.*

As previously stated, it was originally hoped that a study of the refractive indices of the filtrates of samples taken from the serum mixtures from time to time during the heating process would indicate the point at which optimum conditions for filtration had been reached. With this object in view the following procedure was adopted.

Routine concentrations were carried out in which serum or plasma, diluted with one-half its volume of water, and made 30 per cent. of saturation with ammonium sulphate, was heated to a temperature of 61°, 63°, 65° and kept at the specified temperature for a few minutes only, the process taking from 2 to 3 hours in all. Samples of the original plasma and of the plasma mixtures at various stages during the heating process were taken. The samples were filtered and the refractive indices of the clear filtrates were measured by means of the Zeiss Immersion Refractometer. In each case the clear liquid, thus examined, was boiled and filtered and the refractive index of the second filtrate which contained the serum salts and ammonium sulphate in solution, was noted. The amount of protein in solution in the serum mixture was calculated from the difference between the values of the refractive index of the whole serum mixture and of the filtrate containing the serum salts.

TABLE I.

Oxalated plasma diluted with half its volume of water and made 31 *per cent. of saturation with ammonium sulphate. The mixture was heated to* 63° *and kept at that temperature for about five minutes.*

Protein Content of the Original Plasma = 7·45 per cent.

		Scale Divisions of Refractometer Measurements of:		
Duration of time of heating of plasma mixtures	Temperature of mixtures	(*a*) The filtrates from the mixtures	(*b*) The filtrates (*a*) diluted with an equal volume of water, boiled and filtered	Per cent. of protein remaining in solution expressed per 100 cc. of the original plasma
0	16°	98·65	51·50	5·11
1 hr 50′	53·5°	98·72	51·50	5·15
2 hrs 5′	57°	98·55	51·40	5·16
2 hrs 20′	59°	98·56	51·50	5·16
2 hrs 40′	61°	98·50	51·60	5·09
2 hrs 45′	62°	98·85	51·70	5·11
2 hrs 55′	63°	98·90	51·60	5·13

These results have been calculated from the values given in Reiss' table [1904]. For purposes of comparison it has been assumed throughout this research that in serum mixtures containing from 0 to 4 per cent. of protein the difference in protein content for 1 scale division = 0·220 per cent.; from 4 to 8 per cent. the difference for 1 scale division = 0·2175 per cent.

Brailsford Robertson [1915] considers that Reiss' figures are somewhat high, but since in the present investigation the *relative change* only in the protein content of the serum mixture is to be investigated it follows that it is quite immaterial whether Reiss' values are above or below the true value.

An examination of the results from a typical concentration given in Table I shows that during the heating process there was no appreciable change in the refractive indices of filtrates of the serum mixtures heated from room temperature to 63° or 65° and kept at these latter temperatures for

5 or 10 minutes. The very slight changes observed may have been due to the effect of heating the serum itself (*post* p. 301 and Table XIV).

Banzhaf seems to consider that the value of the heating lies in the conversion of soluble pseudoglobulin into a further amount of euglobulin, and that the rapid filtration of the serum mixture depends upon the success of this conversion. Had there been any such change in the amount of protein remaining in solution in the serum mixture, there should have been corresponding variations in the refractive indices of the solution. But, as no change occurred in this physical property of the mixtures one must conclude that a certain amount of euglobulin is thrown out of solution on the addition of the ammonium sulphate at room temperature, and that this amount is not further increased during the heating process.

Since the conversion of pseudoglobulin to euglobulin apparently does not occur during the heating process, it is not improbable that the value of the heating lies in its complete aggregation of the particles of precipitated euglobulin into conglomerates of such dimensions that they are readily retained on the surface of filter paper. The advantage of rapid over slow preliminary heating of the serum mixture—*i.e.* up to 57°—in producing an easy filtering fluid, is most readily explained on the same ground. If the heating were insufficient the smallest particles would pass the filter, while the slightly larger would actually engage and block the pores, producing an ultra filter which would impede or even stop filtration.

With the object of testing the correctness of these hypotheses, the following experiments were accordingly instituted.

Experiment I. Separate volumes of plasma, undiluted or diluted, were made 20, ... 54 per cent. of saturation with ammonium sulphate. The refractive indices of these mixtures were measured and the protein still remaining in solution after the addition of the various amounts of ammonium sulphate solution was calculated, the results, for the purposes of comparison, being expressed in terms of the original plasma.

The various mixtures were then heated to a temperature of 61° or 63° according to the directions given in Banzhaf's paper, and the protein contents of the filtrates from samples of the mixtures were measured at the end of the heating process. The results have been embodied in Tables II, III, IV, and V and in Figure 1.

(*a*) The curve plotted in Figure 1, giving the relation between the concentration of ammonium sulphate in the mixtures and the amount of protein remaining in solution, shows that precipitation occurs mainly at percentages of saturation with ammonium sulphate greater than 28. However the gradient of the curve indicates no critical points for the precipitation of the eu- or pseudo-globulin fraction or of the serum albumins.

(*b*) After heating the mixtures containing concentrations of ammonium sulphate of less than 28 per cent. of saturation, clear liquids could not be obtained—the filtrates remained permanently opalescent and even on long standing the particles of the suspension did not coagulate sufficiently to be retained by filter paper.

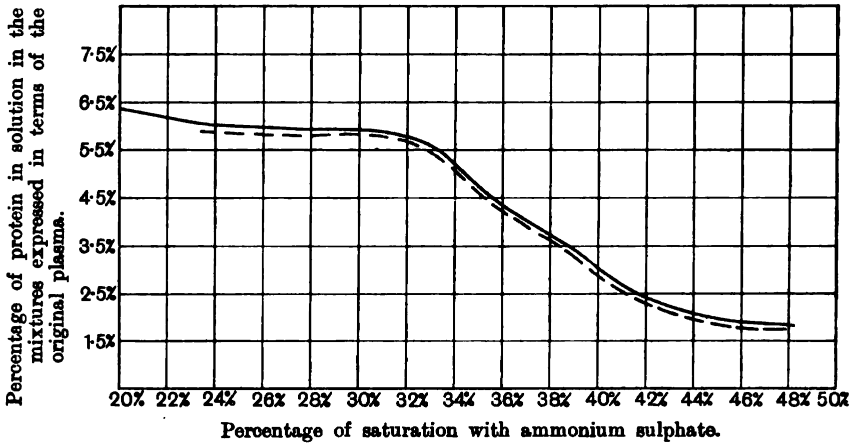

Fig. 1. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate during the process of heating the mixtures just to a temperature of 61° and keeping them at that temperature for a few minutes only.

The curve ——— represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ at room temperature.

The curve — — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ and heated just to 61°.

(*c*) If the mixtures were kept at a temperature of 61° or 63° for a few minutes only, as advocated by Banzhaf, there was, during the heating process, no appreciable change in the protein content of the solution indicated by the refractometer (Tables II and III).

(*d*) If the mixtures were kept at a temperature of 61° or 63° for three-quarters of an hour, or longer (Table IV), then there was, during the heating process, a considerable change in the amount of protein in solution.

(*e*) If *undiluted* plasma were made various percentages of saturation with ammonium sulphate and the mixtures heated to 63° and kept at that temperature for a few minutes only, there was no appreciable change in the protein content of the solutions during the heating process (Table V).

TABLE II.

Oxalated plasma was diluted with half its volume of water and made various percentages of saturation with ammonium sulphate. The mixtures were then heated to a temperature of 61° *and kept at that temperature for a few minutes, the heating process taking two hours in all.*

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma[1]		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	6·49		
20	6·30	6·10	3·2
24	5·91	5·80	1·9
26	5·89	5·79	1·6
28	5·84	5·61	4·0
30	5·85	5·76	1·6
31	5·53	5·36	3·1
32	5·18	5·12	1·1
33	5·18	5·04	2·7
34	4·78	4·69	1·9
36	4·25	4·12	3·1
38	3·84	3·74	2·6
40	2·80	2·86	−2·1
44	2·02	1·90	6
48	1·90	1·90	0

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[1] Throughout these tables it has not been deemed necessary to make corrections for the fact that the addition of x cc. of ammonium sulphate solution to $100-x$ cc. of serum does not necessarily yield a volume of 100 cc. of mixture.

TABLE III.

Oxalated plasma was diluted with half its volume of water and made various percentages of saturation with ammonium sulphate. The mixtures were heated to a temperature of 63° *and kept at that temperature for a few minutes.*

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	8·36		
25	7·35	(Too opalescent to be read)	
28	7·33	6·98	5·0
29	6·81	6·52	4·3
30	6·08	5·92	2·7
32	5·92	5·61	5·3
34	5·48	5·44	0·74
35	5·24	5·11	2·5
40	4·83	4·68	3·1
46	3·39	3·31	2·4
48	2·08	2·14	−3
50	1·31	1·31	0

TABLE IV.

Oxalated plasma diluted with half its volume of water was made various percentages of saturation with ammonium sulphate. The mixtures were heated to 61° *or* 63° *and kept there for three-quarters of an hour.*

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma			Percentage conversion of *soluble* into *insoluble* protein during the heating process in periods	
	(*a*) Before heating	(*b*) After heating just to 61°	(*c*) After 0·75 hr further heating at 61° or 63°	(*b*)	(*c*)
Original plasma	6·49				
30	5·76	5·63	4·28[1]	2·3	25·7
33	5·19	5·02	3·78[2]	3·3	27·2
34	4·81	4·67	3·55[3]	3·1	26·1

[1] Heated to 61° for 1 hour. [2] Heated to 62° for 0·75 hour. [3] Heated to 63° for 0·75 hour.

TABLE V.

Undiluted oxalated plasma was made various percentages of saturation with ammonium sulphate. The mixtures were heated to a temperature of 63° *and kept at that temperature for a few minutes only.*

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	7·14		
28	6·23	5·97	4·2
30	5·98	5·83	2·4
31	5·73	5·43	5·3
32	5·33	4·98	6·6
33	5·17	4·90	5·3

Experiment II. That there was no appreciable change in the pseudoglobulin content of the mixtures during the process of heating was demonstrated gravimetrically as follows.

Several volumes of plasma, both diluted and undiluted, were taken and to them was added respectively ammonium sulphate solution in quantity to make a series of percentage solutions of varying concentration. Each series was duplicated. The same process was carried out with serum.

Of the duplicates of each mixture the one was dialysed without heating and the other previous to dialysis was heated to a temperature of 61° or 63°, and kept there for a few minutes only. After dialysis the residues were made up to a certain volume, aliquot parts of the clear liquid were taken and gravimetric estimations were made on the protein contained therein. Two protein estimations were made on each dialysis residue:

(*a*) The total protein present soluble in saturated brine;

(*b*) The total amount of protein present not precipitated by 50 per cent. saturated ammonium sulphate.

In some cases the total protein in solution in the dialysis residues was estimated gravimetrically and also by means of the refractometer. Gravimetric estimations were invariably slightly higher than those obtained from the refractometer readings, thus indicating that in the dialysis residues there is, in colloidal suspension, a certain amount of insoluble euglobulin, the particles of which are too small to be retained by filtration through paper.

The gravimetric estimations, collected in Table VI, confirm the evidence obtained from the use of the refractometer that there has been no appreciable conversion of pseudoglobulin into euglobulin during the heating process.

The evidence thus afforded in Experiments I and II indicates that Banzhaf's supposition that the heating process in his new method serves to convert some of the pseudoglobulin into euglobulin does not provide such an explanation of the changes taking place as can be verified by quantitative examination.

At first sight it is difficult to reconcile the conclusions thus to be drawn from a study of these data with the obvious fact that, during the heating process of the serum mixture the bulk of the precipitate visibly increases. The following experiment, however, serves to throw light on this apparent paradox.

Experiment III. Serum mixtures were made from 25 to 54 per cent. of saturation with ammonium sulphate and, after standing at room temperature in stoppered flasks for 2 or 3 hours, were filtered. The clear filtrate was divided into two portions and kept in stoppered flasks. In the one portion, which stood at room temperature for several days, the clear liquid, within a few hours, became cloudy, and there was then a deposition of solid protein which gradually increased in bulk. The other portion was heated to 63° (two and a half hours being taken just to reach that temperature) and in every case a precipitate was formed, although at dilutions below 28 per cent. of saturation with ammonium sulphate the precipitate was so

TABLE VI.

Gravimetric estimation of the protein "soluble in saturated brine" in solution in the serum mixtures before and after being subjected to the heating process.

Volume of plasma or serum previously diluted with half its volume of water	Percentage protein content of original plasma	Electrolytes added	Treatment before dialysis	Proportion of mixtures taken for dialysis	Total protein soluble in saturated brine solution		Protein not precipitated by making dialysis residue 50 % saturation with $(NH_4)_2SO_4$	
					(*a*) in the dialysis residue	(*b*) in 100 cc. of original plasma	(*a*) in the dialysis residue	(*b*) in 100 cc. of original plasma
					g.	g.	g.	g.
A. PLASMA (oxalated)	7·14							
(i) 50 cc.	—	made 30 % of saturation with $(NH_4)_2SO_4$	unheated	whole = ppte + liquid	1·324	3·972	0·650	1·950
(ii) 50 cc.	—	„	heated	whole	1·325	3·975	0·649	2·060
B. PLASMA (oxalated)	7·14							
(i) 50 cc.	—	made 32 % of saturation with $(NH_4)_2SO_4$	unheated	„	1·424	4·272	0·635	1·905
(ii) 50 cc.	—	„	heated	„	1·576	4·728	0·6365	1·910
C. PLASMA (oxalated)	5·76							
(i) 50 cc.	—	„	unheated	„	0·995	2·984	0·602	1·806
(ii) 50 cc.	—	„	heated	„	1·015	3·045	0·602	1·805
D. SERUM (carbolised)	5·51							
(i) 50 cc.	—	„	unheated	„	0·678	2·034	—	—
(ii) 50 cc.	—	„	heated	„	0·600	1·800	—	—

finely divided that the liquid could not be filtered clear for refractometer readings. In all cases, whether the filtrates had been heated or not, although there was the visible formation of solid matter from the previously clear filtrate, there was no change in the refractive indices of the solutions, thus indicating no further change in the protein content of the solution after the immediate effect due to the addition of the electrolyte at room temperature. Moreover, this delayed, slowly forming, precipitate was not a special feature of the mixtures containing a percentage of saturation with ammonium sulphate insufficient to precipitate completely the euglobulin from solution, *i.e.* up to 33 per cent. of saturation with ammonium sulphate, for it occurred in all of the ammonium sulphate-serum mixtures.

We are therefore led to no other conclusion than that the effect of heating the serum mixtures just to 61° or 63° merely serves to accelerate a change which takes place slowly at ordinary room temperature, and that whatever this change may be, it is not concerned with the conversion of the pseudoglobulin into the euglobulin.

Light may however be thrown on the question by a consideration of the following observations. Proteins belong to the hydrophile type of colloids, and in solution form an emulsoid system. It has been demonstrated by Chick and Martin [1913] and others that if a high concentration of electrolytes be added to a solution of a protein, there is withdrawal of water from the internal phase of the emulsoid system, and if the withdrawal be sufficient, this phase becomes rigid and is eventually precipitated. In the salting out of proteins the emulsoid is thus first converted into a suspensoid system. Applying these observations to the salting out of serum proteins, it is probable that in the fractional precipitation, with additions of ammonium sulphate to less than 33 per cent. of saturation, the euglobulin is first changed from the emulsoid to the suspensoid type of colloid: the suspensoid is in part converted into a suspension and the latter settles as a precipitate. The protein thus converted into a suspensoid or suspension will no longer have a marked effect on the viscosity or on the refractive index of the solution—even though the particles be neither visible to the naked eye, nor retained by filter paper.

The experimental evidence given above (Experiment III) shows that the complete agglutination of the particles of the suspensoid to those of a perceptible precipitate takes place slowly at ordinary temperatures, and that the effect of heating the liquids in the new process merely serves to increase the velocity of this change. Clearly then, the change thus taking place is not that suggested by Banzhaf, viz. the conversion of pseudoglobulin into euglobulin, but rather a more simple physical process involving the agglutination of the particles of euglobulin already thrown out of emulsoid suspension by the ammonium sulphate. This agglutination permits of easy separation of the euglobulin by filtration through paper.

If, for some reason or other, in the routine concentrations of batches of sera or plasma, the precipitated euglobulin is not completely agglutinated during the heating process, then difficulties will be encountered in the subsequent process of filtration. Some of the insufficiently agglutinated aggregates readily pass through the pores of the filter paper, while others, not sufficiently large to be retained on the surface of the paper, either partially or wholly block the pores of the paper and so retard the process of filtration. (Table VII shows that the partially blocked paper may act as an ultra filter.) The euglobulin thus passing into the filtrate will be precipitated with the pseudoglobulin and antitoxin at one-half saturation with ammonium sulphate. Theoretically speaking, when the precipitated pseudoglobulin and antitoxin

TABLE VII.

Experiment to demonstrate the power of insufficiently agglutinated particles in a badly filtering heated serum mixture to convert an ordinary filter paper into an ultra filter.

Oxalated plasma, diluted with half its volume of water and made various percentages of saturation with ammonium sulphate had been heated just to 61°. The serum mixtures filtered badly; at first there was an opalescent filtrate but the filtration soon slackened and almost ceased.

The protein content of the first opalescent filtrate was estimated and compared with that of the slowly forming clear drops of filtrate collected a short time before the filtration ceased.

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma			The self made ultra filter has effected the removal of soluble protein to the extent of
	(a) Before heating	(b) After heating		
		(i) First filtrate slightly opalescent	(ii) Clear drops just before filtration ceased	
Original plasma	6·49			
24	5·91	5·80	4·68	20·8 %
26	5·89	5·80	4·69	20·3 %
28	5·84	5·61	4·86	16·8 %
30	5·85	5·76	5·30	9·5 %
31	5·53	5·36	5·22	5·6 %

containing a small amount of euglobulin is dialysed until the salt content approaches that of tap water, the pseudoglobulin and antitoxin only should be in solution, but it is generally accepted that the presence of one protein in solution protects another from precipitation.

We have seen (Experiment II) that even in dilute dialysis residues a small amount of euglobulin remained in colloidal suspension. Since, in routine concentrations, the dialysis residues may contain up to and even more than 20 per cent. of pseudoglobulin, the protecting power of the latter in respect of the euglobulin is enhanced. The euglobulin thus in colloidal suspension will greatly retard, if not render impossible, the filtration of the final product through filter candles.

The impediment to filtration in the new process caused by the suspensoid particles of euglobulin was in practice removed by allowing the dialysis residue to stand for a prolonged period. This tedious method has had to be resorted to frequently.

II. *An investigation of the rôle of Sodium Chloride in improving the Banzhaf new One Fraction Method.*

Having ascertained the value of the heating process in the new method, the question then arose as to the part played by the sodium chloride in improving the concentration process. Did the addition of salt to the serum mixtures serve to induce the desired conversion of pseudoglobulin into euglobulin or did it merely exert some stimulating effect on the agglutination of the aggregates of the euglobulin, a process which, from the experimental work in Part I, seems to take place slowly?

In order to elucidate this point, routine concentrations in which 1·5 per cent. of solid sodium chloride had been added to serum or plasma mixtures which had been made 30 per cent. or 31 per cent. of saturation with ammonium sulphate were carefully examined at different stages of the heating process. As before, samples were taken from the mixtures and filtered, and the protein content of the clear filtrates was estimated from the difference between the refractometer readings of the clear filtrate before and after coagulation of the protein therein. For purposes of comparison the protein content of the mixtures at various stages in the heating process was expressed in terms of the original serum or plasma.

Up to the present the concentration of 20 batches each of not less than 50 litres of plasma or serum has been followed; the data with regard to three typical concentrations are given in Table VIII. In no case has any difficulty been experienced in the filtration of the hot liquids or of the final product so far as this latter has up to the present been filtered. In no case has there been any appreciable change in the protein content of the mixtures during the heating process.

From these observations we are led to the conclusion that the effect of the heating process is to agglutinate the particles of euglobulin already converted from the emulsoid to the suspensoid state. This process, which seems to be incomplete in some serum mixtures, is stimulated by the addition of sodium chloride to the same mixtures.

TABLE VIII.

Changes taking place during the heating process to 63° *or* 65° *in the protein content of routine concentration serum mixtures which have been made* 30 *or* 31 *per cent. of saturation with ammonium sulphate and* 1·5 *per cent. with solid sodium chloride.*

Amount of oxalated plasma concentrated	Protein content of original plasma	Percentage of saturation with ammonium sulphate	Duration of time of heating of mixtures	Temperature of mixtures at which samples were taken	Residual percentage of protein in solution in the filtrates of the serum mixtures, calculated from the refractometer readings and expressed in terms of the original plasma
Diphtheria 50 litres	7·00	31	0	Room. temp.	5·11
			2 hrs	57°	5·00
			2 hrs 35′	61°	5·02
			3 hrs	63°	4·93
Tetanus 50 litres	7·47	30	1 hr 55′	57°	6·82
			2 hrs 25′	61°	6·60
			2 hrs 55′	63°	6·60
Tetanus 50 litres	6·73	30	0	Room temp. = 16°	5·19
			1 hr 35′	57°	5·28
			2 hrs 15′	61°	5·28
			2 hrs 35′	65°	5·12

Additional evidence on this point was furnished by the following experiments.

Experiment IV. Serum or plasma (diluted or undiluted) was made from 25–50 per cent. of saturation with ammonium sulphate and solid sodium chloride was added to the mixture to the extent of 1·5 per cent. The protein content of the filtrates of samples of the mixtures was estimated from the refractometer readings as in the previous experiments. The mixtures were then subjected to various treatments.

(*a*) The mixtures were heated to 63° and kept at that temperature for a few minutes, the process in all taking from 2 to 2·5 hours. (Table IX.)

It will be seen from the measurements of the refractive indices of the solutions and from the curves in Figure 2 that there was no appreciable change in the pseudoglobulin content of the mixtures during the process of heating.

(*b*) The mixtures were heated to a temperature of 67° and kept at that temperature for 10 minutes. (Table X and Figure 3.)

TABLE IX.

Oxalated plasma diluted with half its volume of water, made various percentages of saturation with ammonium sulphate and 1·5 *per cent. solid sodium chloride added to the mixtures. The mixtures were heated to a temperature of* 63° *and kept at that temperature for a few minutes.*

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates from the mixtures and expressed, for purposes of comparison, in terms of the original undiluted plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	8·30		
29	6·93	6·66	4·0
30	6·73	6·52	3·1
31	6·38	6·14	3·8
32	5·97	5·89	1·36
33	5·09	4·85	0·8
34	4·81	4·70	2·96
36	3·73	3·64	2·5
39	2·44	2·39	2·1
40	2·19	2·19	0
46	2·15	2·15	0
54	0·574	0·582	– 1·6
Original plasma	7·14		
29	5·68	5·44	4·3
30	5·30	5·25	1
31	5·12	5·08	0·8

From the results it is clear that heating the serum mixtures to this higher temperature is accompanied by a considerable change in the protein content of the solutions.

(*c*) The mixtures were heated to a temperature of 63° and kept at that temperature for 1·25 hours. (Table XI and Figure 3.)

Obviously the prolongation of the time of heating at 63° also gives rise to considerable change in the protein content of the solution.

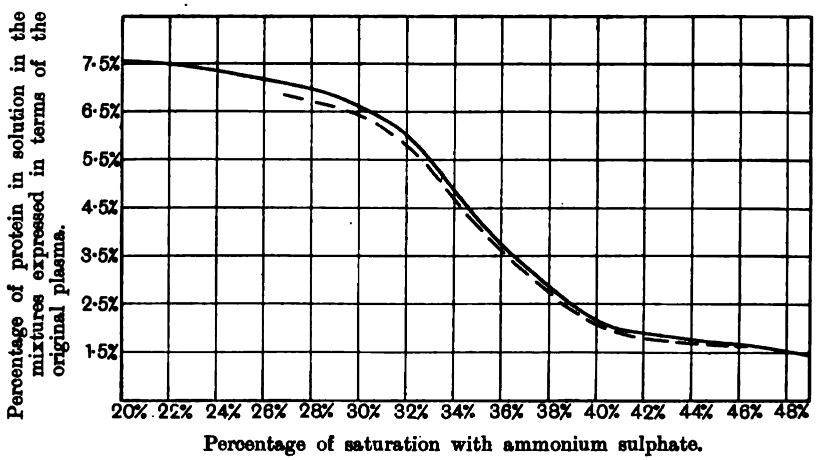

Fig. 2. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate and 1·5 per cent. with solid sodium chloride during the process of heating the mixtures just to a temperature of 63° and keeping them at that temperature for a few minutes only.

The curve ——— represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ at room temperature.

The curve — — — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ heated to 63° and kept at that temperature for a few minutes only.

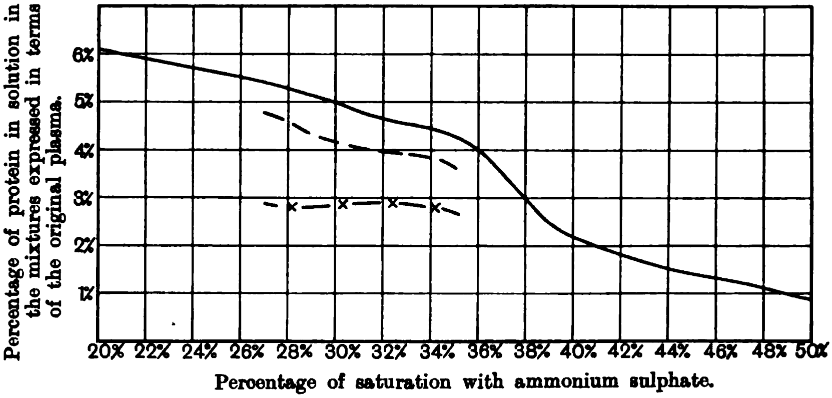

Fig. 3. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate, during the process of heating (*a*) just to a temperature of 67° and (*b*) to a temperature of 63° and keeping at that temperature for 1½ hours.

The curve ——— represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ at room temperature.

The curve — — — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ and heated just to 67°.

The curve — × — × — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ and heated to 63° and kept at that temperature for 1½ hours.

Experiment V. That there was no appreciable change in the pseudoglobulin content of the mixtures during the heating process adopted in the new method was also demonstrated gravimetrically.

Several volumes of serum or plasma, diluted or undiluted, were respectively made various percentages of saturation with ammonium sulphate, and 1·5 per cent. of solid sodium chloride was then added. Each series was duplicated and of the duplicates the one was dialysed without heating and the other, previous to dialysis, was heated to a temperature of 63° and kept at that temperature for a few minutes only—the heating process taking 2 to 2·5 hours in all.

TABLE X.

Oxalated plasma diluted with half its volume of water was made various percentages of saturation with ammonium sulphate and 1·5 *per cent. of solid sodium chloride was added to the mixtures which were then heated to a temperature of* 67°.

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates from the mixtures and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	6·23		
28	5·34	4·66	12·9
29	5·14	4·24	17·7
30	4·98	4·00	20·0
31	4·97	4·25	14·5
32	4·62	3·99	13·6
33	4·54	4·05	11.0
34	4·56	3·96	10·4

The protein estimations were made on each dialysis residue, viz.:

(*a*) The total protein present soluble in saturated brine solution.

(*b*) The total amount of protein not precipitated by 50 per cent. of saturation with ammonium sulphate.

The results showed that, during the heating process, there was no appreciable conversion of pseudoglobulin into euglobulin.

From the results given in Tables VI and XII it is interesting to note that the amount of protein precipitated from the dialysis residues by saturation with brine depends upon the original dilution of the serum or plasma. This is a generally accepted fact (Banzhaf-Gibson).

In certain cases the whole serum mixture, *i.e.* precipitate and liquid, as well as controls of the filtrate alone were dialysed, when it was observed that the pseudoglobulin was much greater in amount in the former than in the latter case: that is to say that the precipitate brought down by the ammonium sulphate was in reality a mixture of eu- and pseudo-globulin.

Further, in those instances in the above experiment in which oxalated plasma was used, the dialysis residues of the unheated plasma mixtures contained the insoluble fibrin and euglobulin formed into a jelly-like clot. In the dialysis residues of the heated mixtures these insoluble proteins were present as a flocculent and readily subsiding precipitate.

Experiment VI. Further experimental work was then undertaken to ascertain the effect of sodium chloride on the solubility of serum proteins under different conditions.

TABLE XI.

Oxalated plasma, diluted with half its volume of water, was made various percentages of saturation with ammonium sulphate and 1·5 *per cent. solid sodium chloride added to the mixtures which were then heated to* 63° *and kept at that temperature for* 1·25 *hours.*

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates from the mixtures, and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	6·23		
28	5·44	2·81	48·6
30	5·29	2·89	45·5
32	4·56	2·80	39·0
34	4·62	2·86	38·4

(*a*) *The effect of heating a saturated brine solution of pseudoglobulin.*

The globulin precipitate thrown down by making plasma 50 per cent. of saturation with ammonium sulphate was suspended in saturated brine solution and after standing at room temperature for 48 hours was gradually heated to a temperature of 61°, the process taking 2 hours in all.

From the use of the refractometer it was ascertained that at room temperature (after 48 hours) the saturated brine contained protein in solution corresponding with a value of 6·3 per cent. in the original plasma. After

TABLE XII.

Gravimetric estimation of the protein "soluble in saturated brine" present in serum mixtures made various percentages of saturation with ammonium sulphate and 1·5 per cent. with sodium chloride, before and after being heated to 63° and kept at that temperature for a few minutes only.

	Percentage protein content of citrated plasma taken	Dilution of plasma	Volume of diluted plasma taken	Percentage of saturation with $(NH_4)_2SO_4$	Treatment of mixtures *previous to dialysis*	Total protein soluble in saturated brine		Protein not precipitated at 50 per cent. of saturation with $(NH_4)_2SO_4$	
						(*a*) the dialysis residue	(*b*) 100 cc. of original serum or plasma	(*a*) in the dialysis residue	(*b*) in 100 cc. of original plasma or serum
A.	7·14	undiluted	100 cc.	29	The mixture, after standing 2 hrs, was filtered. Of the clear filtrate				
					(i) 50 cc. were unheated	1·074	3·024	0·633	1·880
					(ii) 50 cc. were heated	1·098	3·091	0·620	1·745
B.	7·14	undiluted	100 cc.	31	The mixture, after standing 2 hrs, was filtered and of the clear filtrate				
					(i) 50 cc. were unheated	1·596	4·62	0·624	1·800
					(ii) 50 cc. were heated	1·700	4·92	0·600	1·714
C.	7·14	undiluted	2 lots of 50 cc. each	31	The whole mixtures, *i.e.* ppte and liquid, were respectively taken and				
					(i) unheated	2·68	5·36	1·17	2·34
					(ii) heated	2·56	5·12	1·13	2·26
D.	5·76	diluted with ¼ its vol. of H_2O	100 cc.	31	The mixture, after standing 2 hrs, was filtered and of the clear filtrate				
					(i) 50 cc. were unheated	0·234	0·641	—	—
					(ii) 50 cc. were heated	0·227	0·621	—	—

raising the temperature of the brine solution just to 61°, the protein in solution had decreased to the value of 2·48 per cent.

During the heating process there had thus been a conversion of 60·6 per cent. of the soluble into insoluble protein; at the same time there was considerable loss of antitoxic units from the solution.

TABLE XIII.

The effect of the prolonged heating of plasma, diluted with one-third its volume of water and containing 1·5 *per cent. of sodium chloride, upon* (*a*) *the solubility of the serum proteins and* (*b*) *the precipitating power of* 30 *per cent. of saturation with ammonium sulphate.*

		Residual percentage of protein in solution		
Length of time diluted plasma or serum was heated	Temperature of heated liquids	(*a*) in the diluted plasma or serum at various stages in the heating process calculated from the refractometer readings of the heated liquids and expressed, for purposes of comparison, in terms of the original plasma	(*b*) after making the heated liquids 30 per cent. of saturation with $(NH_4)_2SO_4$, calculated as in (*a*)	Percentage increase in the amount of soluble protein precipitated by 30 per cent. of saturation with $(NH_4)_2SO_4$
Plasma (*oxalated*)				
0 hrs	Room temperature	7·38	5·12	—
3 hrs	57°	7·05		
7 hrs	57°	7·01	4·84	5·5
Serum (*carbolised*)				
(1) 0 hrs	Room temperature	6·47	5·82	
15 hrs	57°	5·99	3·81	34·6
(2) 0 hrs	Room temperature	6·95	5·41	
15 hrs	57°	5·77	3·13	42·2

(*b*) *Prolonged heating of serum or plasma to which* 1·5 *per cent. sodium chloride had been added.*

Plasma and serum were each diluted with one-third of their volume of water, 1·5 per cent. of salt added, and heated to a temperature of 57° for periods varying from 6 to 15 hours.

The refractometer readings showed that during the heating process there was a slight change in the protein content of the solutions, but this change did not take place with less than three hours' heating. Samples of the heated mixtures, taken after 6 and 15 hours' heating respectively, were made 30 per cent. of saturation with ammonium sulphate and the protein thrown out of solution compared with that precipitated by the same saturation with ammonium sulphate in the original unheated serum or plasma. (Table XIII.)

The results show that the amount of protein remaining in solution after making the serum 30 per cent. of saturation with ammonium sulphate is practically the same for the unheated serum as for the serum heated to 57° for 6 hours. After 15 hours' heating there is a 30–40 per cent. increase in the precipitation of protein.

Further heating of the respective ammonium sulphate mixtures to a temperature of 61° was not accompanied by any increased change in the protein content of the solutions.

(c) *The heating of serum or plasma without the addition of salt.*

In order to ascertain whether the change thus taking place in (*b*) during the prolonged heating of plasma or serum with 1·5 per cent. sodium chloride was due to the specific action of the sodium chloride on the proteins of horse serum or was an inherent property of the proteins themselves, plasma and serum diluted with water were heated, *without the addition of salt*, in order to ascertain whether changes had been induced during the heating.

(*a*) *Changes in the solubility of the serum proteins.*

During the heating process there was slight deposition of proteins and samples of the heated liquids filtered with difficulty.

From Table XIV it will be seen that whether the serum or plasma had been heated at 63° for a short time or kept at lower temperatures for longer periods there was a slight change in the solubility of the proteins.

(β) *Changes in the precipitating power of* 30 *per cent. of saturation with ammonium sulphate.*

The results depicted in Table XV show that considerable changes may take place in the precipitating power of this reagent.

It has been previously demonstrated that the heating of the serum to 63°, when the liquid was kept at that temperature for a few minutes only, was not followed by any appreciable change in the amount of protein precipitated by 30 per cent. of saturation with ammonium sulphate. But if the liquids were kept at this temperature for 1 hour, or if the temperature were raised to 67°, then considerably more protein was precipitated (from 10 to 20 per cent. increase).

TABLE XIV.

The effect of the heating of serum or plasma on the solubility of the serum proteins
[*No addition of* NaCl.]

Dilution of the oxalated plasma or serum	Temperature to which heated	Duration of the time of heating at the specified temperature	Percentage of protein in solution in the plasma—measured by means of the refractometer		Percentage conversion of *soluble* into *insoluble* protein during the heating process
			(*a*) Before heating	(*b*) After heating	
(1) *Plasma* with ½ its vol. of H_2O	63°	10 min.	7·69	7·43	3·4
(2) *Plasma*	57°	0 hrs	5·85	—	
with ⅓ its vol. of water	57°	15 hrs	5·85	5·30	9·4
(3) *Plasma*	57°	0 hrs	6·14	—	
with ⅓ its	57°	4 hrs	„	5·99	2·4
vol. of water	57°	15 hrs	„	5·79	5·7
(4) *Carbolised serum*[1]					
with ⅓ its	57°	0 hrs	5·59	—	
vol. of water	57°	15 hrs	„	3·79	32·2

[1] A considerable change always takes place during the heating of carbolised serum. This point is being investigated.

TABLE XV.

The effect of the prolonged heating of plasma and of serum respectively diluted with one third their volume of water upon the precipitating power of 30 *per cent. of saturation with ammonium sulphate.* [*No addition of* NaCl.]

Temperature reached	Duration of the heating at the specified temperature	Percentage of protein in solution at the stage in the heating process. Expressed in terms of the original plasma	Percentage of protein remaining in solution after making the liquid 30 per cent. of saturation with ammonium sulphate. Expressed in terms of the original plasma	Percentage change in the amount of dissolved protein precipitated by 30 per cent. of saturation with $(NH_4)_2SO_4$
Carbolised serum				
57°	0 hrs	5·59	4·53	
	15 hrs	3·79	2·42	46·0
Plasma				
57°	0 hrs	5·85	5·80	
	15 hrs	5·30	3·52	39·3
Plasma				
57°	0 hrs	6·14	5·78	
	4 hrs	5·99	5·48	5·2
	15 hrs	5·79	3·52	39·0
Plasma				
55°	0 hrs	7·39	5·35	
	15 hrs	6·89	4·81	10·0

If the plasma or serum were kept at a temperature of 55° for 15 hours there was a 10 per cent. increase in the precipitating power of 30 per cent. saturation with ammonium sulphate.

When the plasma or serum had been kept at 57° for 4 hours there was very little difference between the amounts of protein precipitated from the heated and unheated liquids by the addition of saturated ammonium sulphate to the extent of 30 per cent. of saturation.

After 15 hours' heating at 57° it was found that, on the addition of ammonium sulphate to 30 per cent. of saturation, from 39–47 per cent. more of the soluble protein was precipitated from the heated than from the unheated liquids.

It is thus obvious that the change taking place in the prolonged heating of serum or plasma with 1·5 per cent. sodium chloride in (*b*) is in no way dependent on the presence of the sodium chloride, as the same phenomenon takes place in the prolonged heating of serum or plasma to which no salt has been added.

That changes take place in the prolonged heating of solutions of pseudoglobulin has been shown by Banzhaf [1908] but from the details given in his paper it is difficult to understand the exact procedure adopted by him. Banzhaf suggests that the change is due to the conversion of pseudoglobulin into euglobulin, but from consideration of the work of Hardy [1899], Chick and Martin [1912], and Chick [1914] it is probable that the change taking place in (*b*) and (*c*) above is of the nature of a denaturation of proteins. For, evidently, during the prolonged heating of the serum or plasma, while the actual solubility of the proteins has been little changed their physical properties have become so altered that their precipitability by electrolytes is considerably increased.

In view of these observations it is at first sight not easy to picture the part played by the sodium chloride in rendering the concentration of antitoxic sera by Banzhaf's new process more certain of success. If it were merely a question of the better salting out of the euglobulin by increasing the dehydrating action exerted by the salt, then why should the addition of 1·5 per cent. sodium chloride to the 29 or 30 per cent. of saturation with ammonium sulphate serum mixtures yield a better and more easily filterable end product than that obtained from 31, 32, 33 per cent. or even 34 per cent. of saturation in the absence of sodium chloride.

Now Banzhaf and Gibson [1907] have stated that the salting out of euglobulin is influenced by the dilution of the plasma and they have given what is, in their opinion, the optimum dilution for routine concentrations.

But, that factors other than the dehydrating effect of the salts added to the serum and the dilution of the serum proteins have to be considered, is shown by an examination of the data collected during the progress of this research. (Table XVI.)

TABLE XVI.

The percentage of protein remaining in solution in serum mixtures made 30 *or* 31 *per cent. of saturation with ammonium sulphate.*

Percentage protein content of the original plasma taken	Treatment of the diluted plasma		Residual percentage of protein remaining in solution in the mixtures and expressed in terms of the original plasma
	(*a*) Percentage of saturation with $(NH_4)_2SO_4$	(*b*) Percentage of NaCl in the mixtures	
7·14	30	—	4·93
6·47	„	—	5·85
6·47	31	—	5·53
8·10	„	—	6·29
8·36	„	—	6·39
7·14	„	—	5·74
6·71	30	1·5	5·16
6·73	„	„	4·19
7·38	„	„	4·97
7·14	„	„	5·20
7·38	„	„	5·12
6·38	„	„	5·81
5·51	„	„	4·53
5·76	„	„	5·80
6·04	„	„	5·76
6·23	„	„	5·29
8·32	„	„	6·39
8·32	31	1·5	5·97
7·14	„	„	5·12
7·28	„	„	5·11
7·00	„	„	5·11
6·71	„	„	4·61
7·47	„	„	6·80
7·28	„	„	4·42
6·79	„	„	5·59

It will be seen, that though the ammonium sulphate concentration and the protein concentration of some of the samples were practically identical, yet the amount of protein left in solution was considerably different. Evidently factors operated which had not been controlled.

It is believed that colloidal aggregates are kept in solution by their mutual repulsion which is due to the electric charge they carry. If this charge be sufficiently reduced, then the forces keeping the particles apart diminish; at the iso-electric point the colloids will be most completely precipitated. In other words, the more nearly the hydrogen-ion concentration of the system approaches that of the iso-electric point for a particular protein, the more complete will be the precipitation of that protein.

Therefore, when dealing with the concentration of antitoxic sera on a large scale, we ought not to lose sight of the fact that the reaction of the serum mixtures is probably of more vital importance in the salting out process than has hitherto been recognised in the methods of practical value advocated for the concentration of antitoxic sera. The more nearly the reaction of the serum mixtures approaches the hydrogen-ion concentration at the iso-electric point of euglobulin, not only should there be more rapid and effective filtration, but more constant results should be obtained with regard to the percentage removal of protein from the solution.

In the Banzhaf new One Fraction Method no cognisance is taken of the reaction of the mixture and this may well be the secret of its uncertainty.

The alkalinity of blood is supposed to be due to the partial hydrolysis of certain sodium salts of weak acids, and if this be the case the introduction of a salt like sodium chloride should slightly reduce the alkalinity. It therefore follows that the stimulating effect of the sodium chloride may be due to the fact that it tends to make the hydrogen-ion concentration of the mixtures approach more nearly that required for the optimum precipitation of euglobulin.

In support of this theory it may be mentioned that during the past few months I have had occasion to study the heat coagulation of 350 samples of serum. With some samples the heat coagulation could be readily brought about. With others the formation of a readily filterable coagulum could only be induced after the addition of from 1 to 6 per cent. of sodium chloride, or by the addition of corresponding amounts of a standard solution of acetic acid. The sodium chloride and the acetic acid were interchangeable and apparently had the same effect whether used separately or whether a certain proportion of the one was replaced by the corresponding proportion of the other.

In this connection experiments are being conducted with the object of ascertaining the influence of alteration of the reaction (hydrogen-ion concentration) of the serum mixtures on the precipitation and filtration of the eu- and pseudo-globulin and antitoxin fractions. For, although the desired improvement in filtration may be obtained by the addition of acetic acid to the more alkaline sera we must not lose sight of the fact that, as salt is known to exert a protecting action on antitoxin, it may be better to adjust the reaction of the medium by means of salt rather than by the addition of acid. The results will be dealt with in a future communication.

SUMMARY.

1. The advantages claimed by Banzhaf for his new One Fraction process are sometimes vitiated by the fact that a final product is obtained which, owing to the presence of an opalescent suspension presumably of euglobulin, cannot be filtered except after long standing.

2. The successful preparation of an easily filterable end product depends on the initial heating of the serum mixtures.

3. The heating process advocated in the new method does not cause a conversion of pseudoglobulin into euglobulin but merely serves to complete the aggregation of the euglobulin into particles sufficiently large to admit of separation by filtration.

4. The uncertainty with regard to the successful heating of the serum mixtures can be obviated by a slight modification of the described technique, viz. by the addition of 1·5 per cent. sodium chloride to the serum mixtures.

5. The following observations have been made with regard to the effect of the addition of sodium chloride to the serum mixtures:

(*a*) During the process of heating the mixtures to 63° or 65° the addition of salt does not induce changes in the amount of proteins still remaining in solution, but if the temperature be raised to 67° or if the mixtures be kept for one hour or more at 63°, then a considerable change in the protein content of the solution takes place.

(*b*) If a solution of pseudoglobulin and antitoxin in saturated brine be heated to a temperature of 61° and kept at that temperature for a few minutes only, there is a conversion of 60 per cent. of soluble into insoluble globulin, but at the same time there is loss of antitoxic units.

(*c*) Prolonged heating of serum or plasma diluted with water and containing 1·5 per cent. sodium chloride leads to an increase in the precipitating power of 30 per cent. of saturation with ammonium sulphate. This change, however, is not due to the presence of the sodium chloride. The prolonged heating of diluted plasma or serum gives rise to the same phenomenon.

Banzhaf considers that the change thus produced in the serum is that of pseudoglobulin into euglobulin, but it seems more probable that the prolonged heating serves to denaturate the protein in solution and to make it more readily precipitable by saturation with sodium chloride or by 30 per cent. saturation with ammonium sulphate.

6. It is possible that the addition of sodium chloride exerts a favourable influence on the filtration processes by virtue of an adjustment of the hydrogen-ion concentration of the serum mixtures.

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XXV. THE ESTIMATION OF CALCIUM.

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(*Received May 24th, 1916.*)

Some time ago Professor Andrewes [1915] was engaged in estimating the calcium in the aortas of normal and diseased persons. For that purpose the aortas were calcined until all organic matter was destroyed. After this operation the residue was worked up for the estimation of calcium by a slight modification of Aron's method [1907]. Certain aortas yielded a residue which only dissolved in strong sulphuric acid after many hours' heating. It was to overcome difficulties of this nature that the following method was devised. We found that a calcined residue which only dissolved in sulphuric acid after very prolonged heating would dissolve completely and in a few moments if heated with a solution of phosphoric acid of suitable strength. The calcium in such a solution can be estimated by the usual volumetric and gravimetric methods, which have for their basis the precipitation of calcium oxalate.

THE METHOD.

Of a normal organ such as heart, or muscle, 100 grams of the substance are required; of an aorta rich in calcium very much less will suffice; of urine 100 cc. is a suitable quantity. The organ or tissue is dried at 100° and incinerated in the usual way; the urine is evaporated at first on the water bath, then dried at 120° and incinerated as before. To the residue a solution of phosphoric acid is added: if much calcium is present, 20 cc. of an acid of sp. gr. 1·2 (1 vol. syrupy phosphoric acid to 3 vols. water): if little, 10 cc. or even less. On warming, the calcium dissolves completely. This solution can be used direct for a volumetric determination, but it must be filtered and the residue washed for a gravimetric determination. The liquid is diluted with 50 or 100 cc. of water according to the amount of calcium present and then a solution of oxalic acid containing 37·8 g. $C_2H_2O_42H_2O$ per litre is added so as to afford about five times the theoretical amount of oxalic acid. Thus in practice if the amount of calcium is about 0·2 g. reckoned as calcium carbonate, 100 cc. of the oxalic acid would be added. Precipitation is rapid, and the precipitate is crystalline, but a little calcium remains in solution. To effect complete precipitation a volume of ammonia equal to the volume of the oxalic acid used is then added, the strength of the ammonia being exactly equivalent to that of the oxalic acid, namely 10·2 g. of NH_3 per litre. If magnesium is present the ammonia must be added slowly and with shaking from a burette. The precipitated solution is allowed to stand for one hour: it will then filter quickly and perfectly clear. If ammonium oxalate be used, instead of oxalic acid followed by ammonia as above, the precipitate is far less crystalline and filters very badly.

A. *Volumetric Determination.*

In this method we make use of a small conical Buchner funnel so as to minimise the amount of washing needed to remove ammonium oxalate from the filter paper. Seven washings with cold distilled water are sufficient for this. The precipitate and paper are transferred to the same beaker in which the precipitation was effected; 10 cc. of sulphuric acid (1 vol. water : 1 vol. pure concentrated acid) are added; the solution heated nearly to boiling and titrated with decinormal permanganate. The sulphuric acid sometimes consumes a few drops of permanganate so that a blank experiment should be made.

B. *Gravimetric Determination.*

This is done in the usual way and needs no description.

RESULTS.

As a check on the method we determined the percentage purity of a sample of commercial calcium carbonate sold as "Calcium Carbonate Reagent."

4·84 g. were dissolved in 25 cc. of syrupy phosphoric acid and made up to 250 cc. with water: 10 cc. were taken, and 50 cc. of water, 100 cc. of oxalic acid and 100 cc. of ammonia were added in every case.

Volumetric Results.

N/10 permanganate required	Percentage $CaCO_3$ found
39·8 cc.	—
39·7 „	102·6
39·8 „	—

Gravimetric Results.

Weight of CaO obtained	Percentage $CaCO_3$ found
0·1104	101·8
0·1104	101·8
0·1102	101·5

By solution of the carbonate in excess of hydrochloric acid and back titration by standard caustic soda we obtained in three experiments 100·64, 101·08, 101·25.

The sample evidently contained more calcium than corresponds to the formula $CaCO_3$: we found in fact that it contained some calcium hydroxide. By solution of some of the carbonate in hydrochloric acid and precipitation with ammonium carbonate, we obtained a specimen of really pure calcium carbonate which gave us a percentage of 100·14.

We now give examples of the determination of calcium in various substances by our own method and those of Aron [1907] and McCrudden [1910, 1911].

Urine. 100 cc. used in each experiment.

Percentage CaO.

Our method	Aron's method	McCrudden's method
0·072	0·071	0·074
0·045	0·044	0·045
0·033	0·032	0·032
0·041	0·033	0·040

Aorta. Portions of the same aorta were used for the determinations by us and by Professor Andrewes working independently.

Percentage CaO in dried Aorta.

	Our method	Andrewes by Aron's method
I	1·31	1·29
II	8·06	7·45
III	1·91	1·72

Calcium in the presence of Magnesium.

To test the validity of the method where calcium is accompanied by magnesium we determined these two elements in solutions, which contained a constant amount of calcium as oxide, and half, once and twice as much magnesium as oxide. The calcium was determined as described and the filtrate and washings from the calcium oxalate were concentrated to about 300 cc., 30 cc. of concentrated ammonia added and the mixture allowed to stand over night. The ammonium magnesium phosphate was then filtered off, washed, dried, ignited and weighed. Our first experiments were made with mixtures of calcium and magnesium phosphate and it appeared as if the magnesium were partly precipitated with the calcium oxalate for, as the proportion of magnesium rose from nought to twice the calcium, the amount of calcium increased. On testing the magnesium phosphate it was found to contain calcium. The use of phosphates was therefore abandoned and solutions of calcium and magnesium oxides in phosphoric acid were used. Of the calcium solution 10 cc. were used and 50 cc. of water added; but when calcium and magnesium were present together, 10 cc. of calcium solution and 150 cc. of water were used with 10, 20, and 30 cc. of the magnesium solution.

Results.

cc. CaO	cc. MgO	CaO found		MgO found
		Volumetric	Gravimetric	
10	0	0·1014	—	—
10	0	0·1014	—	—
10	0	—	0·1018	—
10	0	—	0·1015	—
0	10	—	—	0·0537
10	10	0·1014	—	0·0536
10	10	0·1015	—	0·0532
10	20	0·1030	—	0·1081
10	20	0·1025	—	0·1081
10	30	0·1028	—	0·1625
10	30	0·1025	—	0·1627
10	30	—	0·1030	0·1625

The table shows that when the amount of magnesium as oxide exceeds the amount of calcium as oxide a little of the former is precipitated with the latter metal. In this case the precipitate of calcium oxalate is ignited, redissolved in a little phosphoric acid and precipitated again. This has always given us correct results.

The ease with which calcium compounds dissolve in phosphoric acid might find application in several ways. For example we find that this acid enables us to make an excellent separation of calcium and barium sulphates; calcium sulphate readily dissolves but barium sulphate is quite insoluble in this acid.

Again calcium fluoride is soluble in the acid on heating. We have not examined this reaction quantitatively, but on testing the filtered solution with oxalic acid followed by ammonia abundance of typical calcium oxalate crystals were seen when the precipitate was examined under the microscope.

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XXVI. EXPERIMENTS ON THE INTERACTION OF HYDROXY- AND AMINO-ACIDS WITH NITROPHENYLHYDRAZINE WITH SPECIAL REFERENCE TO THE FORMATION OF GLYOXAL DERIVATIVES.

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(*Received May 26th, 1916.*)

Dudley and the writer [1913, 1914] have shown that when α-hydroxy-acids, such as glycollic, lactic, glyceric or mandelic acid, are digested in aqueous solution with *p*-nitrophenylhydrazine, small amounts of substances identical in properties with the bis-nitrophenylhydrazones of glyoxals are formed. Amino-acids, such as glycine, alanine, valine, leucine, aspartic acid and phenylalanine, in feebly acid solution under similar circumstances gave the same products. The course of the reaction was crudely represented as follows:

$$R \cdot CHOH \cdot COOH \rightleftarrows R \cdot CO \cdot CHO + H_2O$$

$$R \cdot CHNH_2 \cdot COOH \rightleftarrows R \cdot CO \cdot CHO + NH_3$$

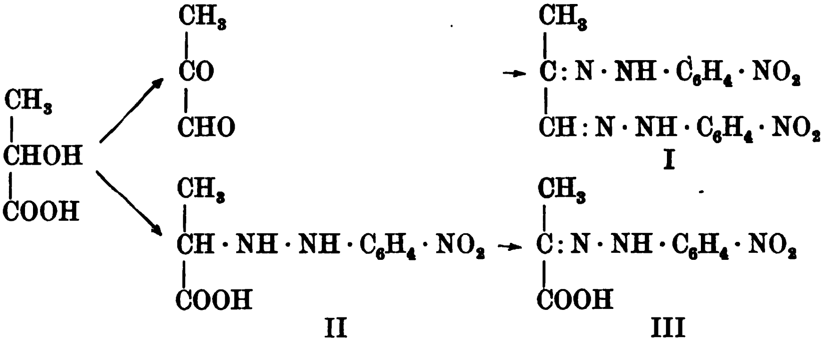
But it should be recalled that the direct synthesis, outside the body, of amino-acids from glyoxals by the action of ammonia or similar substances was never observed.

The qualitative demonstration of the reactions upon which these conclusions are based is, in some respects, easy to carry out, but the yield of glyoxal derivatives is extremely small and their bis-nitrophenylhydrazones are not readily converted into other derivatives and moreover the reactions are complicated by numerous side changes.

The object of the following paper is to record a few additional experiments which throw some light on the reaction. These experiments are manifestly incomplete but as the opportunity to carry them further is at the moment lacking, it appeared worth while to describe them now.

(I) *Formation of* p-*nitrophenylhydrazides from α-hydroxy-acids.*

It has already been shown that when dilute lactic acid is digested with *p*-nitrophenylhydrazine, the following products are formed—(i) methylglyoxal bis-nitrophenylhydrazone, (ii) α-nitrophenylhydrazinopropionic acid, (iii) pyruvic acid nitrophenylhydrazone. This last substance was derived from the hydrazino-acid (ii), from which it is readily obtained by oxidation.



The amount of these three products was however not nearly enough to account for the amount of nitrophenylhydrazine which disappeared. It has now been found that a fourth product, namely, the *p*-nitrophenylhydrazide of lactic acid, $CH_3 \cdot CHOH \cdot CO \cdot NH \cdot NH \cdot C_6H_4 \cdot NO_2$, is formed in relatively large amounts. The substance possesses attractive properties and is easily prepared. A similar crystalline derivative was prepared from *d*-lactic acid and from glycollic acid, while α-hydroxybutyric acid and β-hydroxybutyric acid gave only oily products.

Glycollyl-p-*nitrophenylhydrazide,* $CH_2OH \cdot CO \cdot NH \cdot NH \cdot C_6H_4NO_2$. Glycollic acid (2 g.), nitrophenylhydrazine (4 g.), water (20 cc.) and alcohol (20 cc.) were warmed on a water bath, then diluted with 100 cc. of water and allowed to stand overnight. Most of the unchanged nitrophenylhydrazine was precipitated by adding 2 cc. of saturated sodium acetate solution and the remainder was extracted from the filtrate by shaking with three successive portions of chloroform. The hydrazide was next separated by repeated extraction with amyl alcohol. The latter solvent was removed by evaporation at a low temperature under diminished pressure and the product (3·1 g.) was recrystallised from boiling alcohol in which it is only moderately soluble. The substance crystallises from alcohol in lemon yellow needles melting at 192°–194°. It is readily soluble in water, very sparingly soluble in chloroform or benzene and is easily hydrolysed on boiling with acids. On analysis the substance gave 20·1 per cent. nitrogen compared with 19·9 for the calculated amount.

i-*Lactyl*-p-*nitrophenylhydrazide*. Lactic acid (8·5 g.), *p*-nitrophenylhydrazine (17 g.) and 95 per cent. alcohol (100 cc.) were heated on the water bath in a loosely corked flask for a couple of hours, a little more alcohol was then added and the mixture allowed to stand overnight in an incubator at 37°. Most of the alcohol had then evaporated and the mixture was diluted with half a litre of water. A small precipitate of unchanged base was filtered off and the rest was removed by three extractions with chloroform. The hydrazide was then extracted by repeated shaking with amyl alcohol. The extract was evaporated under diminished pressure and the solid residue of crude hydrazide (15 g.) was recrystallised from a mixture of alcohol and toluene. The yield was 12 g.

In another experiment the reaction was carried out in aqueous solution with excess of lactic acid. Nitrophenylhydrazine (2 g.) was heated for two hours on the water bath with lactic acid (5 cc.) and water (45 cc.). After standing overnight the solution was partly neutralised by adding sodium carbonate until a pink colour transitorily appeared and then successively extracted with chloroform and amyl alcohol as before. The solid crystalline residue was dissolved in alcohol (5 cc.) and toluene added to the warm solution to a point just short of the appearance of turbidity. A yield of 2·1 g. of the hydrazide was obtained.

Lactyl-*p*-nitrophenylhydrazide is an almost colourless substance when pure and crystallises from alcohol in prisms and plates which sinter at 162° and melt at 165°–167°. It is readily soluble in water, alcohol and acetone, sparingly soluble in ether, chloroform and toluene, insoluble in light petroleum. On analysis the substance gave 18·6 per cent. nitrogen compared with a calculated value of 18·7 per cent.

d-*Lactyl-nitrophenylhydrazide*. This substance was prepared from *d*-lactic acid in the same way as the inactive variety. Two g. of the acid and 3 g. of the hydrazine gave 2·1 g. of product crystallising from alcohol and toluene in fine prisms melting at 161°–163° with slight previous sintering. On analysis the substance gave 18·9 per cent. nitrogen. The substance is strongly laevorotatory. Its rotation was observed in absolute alcohol solution.

$$c = 2{\cdot}35, \qquad l = 2 \text{ dm}, \qquad a = 1{\cdot}37^\circ,$$

$$[a]_D^{17^\circ} = -\,34{\cdot}5^\circ.$$

Another preparation had $[a]_D^{17^\circ} = -\,33{\cdot}6^\circ$.

(II) *Conditions affecting the formation of methylglyoxal derivatives and other substances from lactic acid and nitrophenylhydrazine.*

It has been shown that when aqueous lactic acid is digested with *p*-nitrophenylhydrazine at least four well characterised substances are found in addition to indefinite decomposition products arising from the hydrazine. The results of some preliminary experiments relating to the formation of these compounds may be referred to. The products of the reaction were separated in a roughly quantitative fashion as follows. The mixture of aqueous lactic acid and nitrophenylhydrazine prepared as described in previous papers [1913, p. 130], after digestion at 37° for a determined period, was filtered through a weighed Gooch crucible. The insoluble part consists of methylglyoxal-bis-nitrophenylhydrazine with *a*-nitrophenylhydrazinopropionic acid and the nitrophenylhydrazone of pyruvic acid. The last two acid substances were separated by washing the precipitate with hot sodium carbonate solution and recovering them from the alkaline solution by acidifying with acetic acid. The very sparingly soluble methylglyoxal-bis-nitrophenylhydrazone was then washed with water and alcohol. The original aqueous solution after filtering off the precipitate was examined for unchanged hydrazine by acetone while lactyl-nitrophenylhydrazide was separated by partial neutralisation with sodium carbonate, half saturation with ammonium sulphate and extraction with amyl alcohol.

It does not seem of value to record a number of these experiments in detail but the following definite points were established.

(*a*) If the lactic acid is completely neutralised with sodium hydroxide before digestion with nitrophenylhydrazine no formation of methylglyoxal-bis-nitrophenylhydrazone is observed. A free carboxyl group is apparently necessary for methylglyoxal formation.

(*b*) Mineral acids added in small amounts (e.g. 2 per cent. sulphuric acid) to lactic acid solutions (10 per cent.) with nitrophenylhydrazine (0·5 per cent.) inhibit phenylhydrazide formation and slightly diminish the yield of methylglyoxal derivative. The yield of hydrazino-acid is not materially influenced.

(*c*) When aqueous lactic acid and nitrophenylhydrazine are digested together at 37°, a low concentration of lactic acid (e.g. 2 per cent.) favours both the formation of methylglyoxal-bis-nitrophenylhydrazone and of the hydrazino-acid but is unfavourable to the formation of lactyl-nitrophenylhydrazide. Concentrated solutions (e.g. 10 per cent. and over) favour hydrazide formation but are prejudicial to the production of the other products.

(*d*) Formation of methylglyoxal-bis-nitrophenylhydrazone is not conditioned by the prior formation of lactyl-nitrophenylhydrazide since the latter substance could not be converted into the former.

As an example of the relative yields of products the following experiment may be cited. Lactic acid (300 cc.), water (300 cc.) and *p*-nitrophenylhydrazine (30 g.) were rapidly heated to boiling, cooled and filtered. The mixture was divided into three parts; (*a*) was digested undiluted at 37°, the others, (*b*) and (*c*), were diluted with one and three litres of water respectively and digested for ten days. After three days practically all unchanged hydrazine had disappeared from (*a*) while a little more remained in (*b*) and (*c*). The yields of methylglyoxal-bis-nitrophenylhydrazine from (*a*), (*b*) and (*c*) were respectively 31, 77 and 203 milligrams. The yields of α-nitrophenylhydrazino-propionic acid were 116, 482 and 808 milligrams respectively. Almost the whole of the remaining nitrophenylhydrazine was converted into lactyl-nitrophenylhydrazide.

(III) *Glyoxal derivatives from other acids.*

As stated in the introduction, evidence of the formation of glyoxal derivatives has been observed in the case of a number of α-hydroxy- and α-amino-acids when digested with nitrophenylhydrazine. Since the yield of these glyoxal derivatives is very small, it appeared desirable to make comparative experiments with other acids, in order to see if confirmatory evidence could be obtained as to the character of the reaction under investigation.

The following experiments were simply of a qualitative kind. In order to detect glyoxal formation the precipitates obtained on digestion with nitrophenylhydrazine were filtered off and washed first with hot sodium carbonate solution and then with alcohol. The residue was then tested with caustic soda solution and alcohol. A clear blue colour resembling that of Fehling's solution is taken as a probable indication of glyoxal formation since all bis-nitrophenylhydrazones yield this extremely sensitive reaction. Brown, reddish or violet colourations such as many nitrophenylhydrazine derivatives yield are not obtained if the glyoxal derivatives are properly purified with sodium carbonate and alcohol.

The solutions were usually prepared by warming together 4 g. of the acid, 40 cc. of water and 0·2 g. nitrophenylhydrazine. The solutions were rapidly cooled, filtered and digested at 37°. After two or three days any precipitate was filtered off, and examined as described. The results were as follows:

Acid		Reaction of product with sodium hydroxide
Butyric acid	$CH_3 \cdot CH_2 \cdot CH_2 \cdot COOH$	–
α-Hydroxybutyric acid	$CH_3 \cdot CH_2 \cdot CHOH \cdot COOH$	+
β-Hydroxybutyric acid	$CH_3 \cdot CHOH \cdot CH_2 \cdot COOH$	–
Crotonic acid	$CH_3 \cdot CH{=}CH \cdot COOH$	?
Isobutyric acid	$(CH_3)_2 \cdot CH \cdot COOH$	–
α-Hydroxy-isobutyric acid	$(CH_3)_2 \cdot COH \cdot COOH$	+
α-Methoxy-isobutyric acid	$(CH_3)_2 \cdot C(OCH_3) \cdot COOH$	–
Diethylglycollic acid	$(C_2H_5)_2 \cdot COH \cdot COOH$	+
Tartaric acid	$COOH \cdot CHOH \cdot CHOH \cdot COOH$	+
Gluconic acid	$CH_2OH \cdot (CHOH)_4 \cdot COOH$	+

The product from crotonic acid gave a blue colour with caustic soda typical of glyoxal derivatives but the substance differed from the latter in being readily decomposed by hydrochloric acid. It appears likely that some substance with two adjacent nitrophenylhydrazine groups was formed but its structure is quite obscure.

(IV) *Action of semicarbazide on leucine.*

In all the experiments hitherto recorded on the formation of glyoxals from α-hydroxy- and α-amino-acids use has been made of nitrophenylhydrazine as being peculiarly suitable on account of the extraordinarily low solubility of its glyoxal derivatives. Dudley and the writer have shown that isobutylglyoxal gives a fairly insoluble compound with semicarbazide [1914, p. 38]. Accordingly an attempt was made to isolate isobutylglyoxal semicarbazone on long digestion of leucine with semicarbazide sulphate. Unfortunately the experiment gave a negative result. Whether this is due to a too great solubility of the semicarbazone or whether glyoxal formation is in some way conditioned by the presence of nitrophenylhydrazine is not clear. Further work is needed to determine these points. In any case it should be recalled that the simple equation given at the commencement of this paper to represent the dissociation of α-hydroxy- and α-amino-acids is adopted for convenience in explaining certain experimental results rather than on account of a belief in its precise accuracy. Many other alternative forms of dissociation are conceivable.

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XXVII. THE OXIDATION OF AMINO-ACIDS TO CYANIDES.

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The oxidation of α-amino-acids to aldehydes may be effected with a variety of reagents including hydrogen peroxide [Dakin, 1906], lead dioxide and dilute sulphuric acid [Liebig, 1849], alloxan [Strecker, 1862; Hurtley and Wootton, 1911], glyoxals [Dakin, 1914], and also with sodium hypochlorite [Langheld, 1909]. In all of these and many similar reactions, the nitrogen of amino-acids seems very easily removed by oxidation. The action of sodium hypochlorite on amino-acids was carefully studied by Langheld who showed that in all probability the first stage in the reaction was conditioned by the formation of a monochloroamino-acid, which subsequently underwent decomposition as follows:

$$R\cdot CH(NH_2)\cdot COOH \xrightarrow{+NaClO} R\cdot CH(NHCl)\cdot COONa \xrightarrow{+H_2O} R\cdot CHO + NH_3 + NaCl + CO_2.$$

In the case of leucine Langheld isolated the monochloroamino-acid as an unstable solid and obtained good evidence for the formation of a dichloroamino-acid when excess of hypochlorite was used. Apparently he did not investigate the decomposition products of the dichloroamino-acids.

Recently the writer has been engaged with the investigation of the properties of sulphochloroamides such as sodio-*p*-toluenesulphochloroamide, a substance prepared by the combination of sodium hypochlorite and toluene-sulphonamide, and now used to some extent as an antiseptic under the name chloramine-T. It has already been shown that though a solution of this substance contains no free hypochlorite, it reacts with α-amino-acids much in the same way as hypochlorite, yielding aldehydes, carbon dioxide and ammonia [Dakin, Cohen, Daufresne and Kenyon, 1916]. The corresponding aldehydes were prepared from glycine, alanine, leucine, aminophenylacetic acid and methylaminophenylacetic acid.

Now sodio-*p*-toluenesulphochloroamide gives a practically neutral solution and the products of its decomposition are neutral. It appeared therefore that this substance might prove to be a useful neutral oxidising agent suitable for the oxidation of rather unstable substances. Langheld believed that he had obtained a small amount of glyoxaline acetaldehyde from histidine by oxidation with hypochlorite but the characterisation of the substance left much to be desired. It was decided, therefore, to try the action of sodio-*p*-toluenesulphochloroamide on histidine in the hope of obtaining more favourable results. A considerable yield of a crystalline product was obtained which was at first thought to be the aldehyde but which on investigation proved to be cyanomethyl-glyoxaline, a substance already prepared by Pyman [1911]. At first this appeared to be an anomalous result but it now appears that the reaction is of a fairly general character and that cyanides may be obtained by the oxidation of a variety of amino-acids. Apparently the cyanides are produced by the decomposition of previously formed dichloroamino-acids:

$$R \cdot CH(NH_2) \cdot COOH \rightarrow R \cdot CH(NCl_2) \cdot COOH \rightarrow R \cdot CN + 2HCl + CO_2.$$

On applying the reaction to other amino-acids, traces of hydrocyanic acid were obtained from glycine, acetonitrile from alanine, isobutyl cyanide (isovaleronitrile) from leucine and cyanobenzene (benzonitrile) from aminophenylacetic acid. The yields of cyanide in the case of the last three amino-acids were large.

As a matter of fact the reaction is not entirely novel, for Langheld apparently overlooked the fact that Schwanert [1857] many years ago before the constitution of leucine was settled had obtained clear proof of the formation of isobutyl cyanide when alkaline solutions of leucine were treated with chlorine. Liebig [1849] also stated that leucine on treatment with manganese dioxide and sulphuric acid gave isobutyl cyanide, while glycine gave hydrocyanic acid.

In this connection Plimmer's [1904, 1, 2] observation of the formation of hydrocyanic acid from glycine and other amino-acids on oxidation with nitric acid may be recalled; also the production of isobutyl cyanide from gelatin and caseinogen on oxidation with chromic acid recorded by Schlieper [1846] and Guckelberger [1847].

The oxidation of *a*-amino-acids to cyanides may have more than a theoretical interest in certain cases since it affords an additional method for the preparation of amines from amino-acids. Histidine for example may be oxidised to cyanomethyl-glyoxaline and then reduced to β-aminoethyl-glyoxaline without much difficulty and with relatively good yields.

EXPERIMENTAL.

Glycine and Alanine. The production of formaldehyde and acetaldehyde from glycine and alanine (1 mol) when their aqueous solutions are warmed with sodio-*p*-toluenesulphochloroamide (1 mol) has already been recorded [Dakin and colleagues, 1916]. When two molecular proportions of the sulphochloroamide are used to one of glycine and the mixture distilled, the distillate contains traces of hydrocyanic acid which are easily recognisable by the customary tests. In the case of alanine the first portion of the distillate contains oily drops of acetonitrile which subsequently dissolve as the distillate becomes more dilute. A trace of chloroamine, NH_2Cl, is also usually present and is easily recognised by its irritating odour. It disappears on standing owing to decomposition. The acetonitrile is readily shaken out with ether and gives a crystalline hydrobromide sparingly soluble in dry ether. On hydrolysis with sulphuric acid the formation of acetic acid can easily be recognised.

Leucine. The synthetic amino-acid (3·6 g.) was gently warmed with a solution of sodio-*p*-toluenesulphochloroamide (13 g.) in 100 cc. of water and the mixture was then distilled. The oily layer consisting largely of isobutyl cyanide was separated and shaken with sodium bisulphite solution to remove a little isovaleric aldehyde. The cyanide was then taken up in a little ether and distilled from a small distilling flask. 1·8 g. of isobutyl cyanide were obtained corresponding to a yield of about 70 per cent. of the theoretical amount. The cyanide was hydrolysed by boiling under a reflux condenser with excess of fifty per cent. sulphuric acid, the mixture diluted with water and the isovaleric acid recovered by distillation. It was converted into the silver salt which was analysed.

0·5944 g. gave 0·3081 g. silver = 51·8 per cent. Ag
$C_5H_9O_2Ag$ requires 51·7 „ „ „

a-Amino-phenylacetic acid. This acid was oxidised with two molecular proportions of the sulphochloroamide in the same fashion as in the preceding experiment with leucine. The oily distillate consisting of cyanobenzene mixed with some benzaldehyde was dissolved in a little ether, shaken vigorously. with sodium bisulphite to remove benzaldehyde and then the ether layer washed successively with sodium carbonate solution and water. On evaporation of the ether a 60 per cent. yield of crude cyanobenzene was obtained. Part of this was hydrolysed with sulphuric acid to benzoic acid, M.P. 120°, and another portion was converted into benzamide by cautious treatment with alkali. The most satisfactory identification was effected by dissolving 0·5 g. of the oil in 3 cc. absolute alcohol, then adding 3 cc. of concentrated ammonia and saturating the mixture with sulphuretted hydrogen. The solution was then heated in a sealed tube at 100° for an hour, the alcohol evaporated off and the solid residue of thiobenzamide crystallised from boiling water. The yield of thiobenzamide was practically quantitative as stated by Gabriel [1890] and the substance crystallised from water in long fine felted needles melting at 117°.

Histidine. The material for the experiments with histidine was kindly furnished by Dr Ewins. The dihydrochloride (1 g.) was dissolved in 20 cc. of water, neutralised with 0·5 g. of sodium carbonate, and 2·6 g. of sodio-toluenesulphochloroamide was then added. After standing for some time the solution was filtered to remove toluenesulphonamide and the filtrate shaken a few times with ether to remove the bulk of any dissolved sulphonamide. This procedure involves some loss of the nitrile but this can be recovered by washing the extracted sulphonamide with a little water. The main filtrate was then concentrated, preferably under reduced pressure, and before taking to complete dryness excess of dry sodium carbonate was added. The dry residue was then extracted with boiling ethyl acetate. It was found most convenient to do this in a Soxhlet apparatus after mixing the residue with plaster of Paris. The ethyl acetate extracts the cyanomethyl-glyoxaline almost exclusively and on evaporation of the solvent the substance readily crystallises, but it is usually somewhat pigmented As much as 0·4 g. of the cyanomethyl-glyoxaline can be obtained from 1 g. of histidine dihydrochloride corresponding to about eighty per cent. of the theoretical amount.

The cyanomethyl-glyoxaline was purified either by direct crystallisation from water, or from a mixture of methyl alcohol and toluene, or it was converted into the sparingly soluble picrate or finally it was sublimed at a temperature just below its melting point under the very low pressure obtained with the Gaede pump. However obtained the substance melts sharply at 138° and has all the properties described by Pyman [1911].

A mixed melting point with material kindly supplied by Dr Pyman showed no change.

Part of the substance was converted into the picrate which melted sharply at 167° after recrystallisation from water. It was analysed for picric acid by means of Busch's "nitron" method [1905].

0·6148 g. picrate gave 0·9891 g. precipitate = 68·1 per cent. picric acid
$C_5H_5N_3 \cdot C_6H_2(NO_2)_3OH$ requires 68·1 „ „ „

The cyanomethyl-glyoxaline gave the abnormal diazo-reaction and yielded a very soluble hydrochloride agreeing exactly with Pyman's description.

A portion of the cyanomethyl-glyoxaline was reduced to aminoethyl-glyoxaline with sodium and alcohol. The physiologically active base gave the characteristic dipicrate melting at 238°.

The above experiments were carried out in the Biochemical Laboratories of the Medical Research Committee under a grant from the Committee and I am indebted to Drs Dale and Barger for facilitating the work in every way possible.

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XXVIII. THE VISCOSITY AND HYDRATATION OF COLLOIDAL SOLUTIONS.

By EMIL HATSCHEK.

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In a paper published in this *Journal* Miss Chick [1914] has calculated the hydratation factors for a number of proteins from the author's formula for the viscosity of emulsoid sols. By means of this formula the volume of the disperse phase, i.e. the hydrated protein, is obtained from the relative viscosity, and the hydratation or association factor is then found by comparing this volume with the weight of dissolved substance. Miss Chick's results are: (1) that for each protein the association factor is constant over a considerable range of concentration, and (2) that its value lies between 1·57 for crystallised egg albumin and 8·63 for sodium caseinogenate, these figures being the numbers of grams of water associated with each gram of protein in solution.

In a recent paper under the above title Arrhenius [1916] criticises these results and considers them open to grave doubt, chiefly on the ground that the factors are very much larger than those determined (by other methods) in the case of the most highly hydrated salts in solution, such as $MgCl_2 + 12H_2O$ (equivalent to about 2·3 g. of water per g. of salt), which hydrate is known only at − 16·8°, while the viscosity figures for proteins were obtained at a temperature of 25°.

Arrhenius then applies to the viscosity figures treated by Miss Chick his well-known formula:

$$\log \eta = \theta C, \qquad \text{.....(1)}$$

in which C however now means the molecular concentration, while θ is a constant and η the relative viscosity. For substances like proteins, whose molecules are extremely large compared with those of water, he considers that C is expressed with sufficient accuracy by the equation

$$C = \frac{100p}{100 - (n+1)\,p}, \qquad \text{.....(2)}$$

in which p is the number of g. of solute in 100 g. of solution, and n the hydratation factor, i.e. the number of g. of solvent associated with each g. of solute, and therefore withdrawn from the free solvent.

The complete expression for the logarithm of the viscosity of a solution, in which the molecular weight of the solute is high compared with that of the solvent, is accordingly:

$$\log \eta = \theta \frac{100p}{100-(n+1)p}, \qquad \text{.........................(3)}$$

and as this expression contains only two constants, θ and n, it is obvious that these can be calculated from two sets of experimental data. Solving equation (3) for $(n+1)$ we obtain:

$$n+1 = \frac{100}{p} - \frac{100\theta}{\log \eta}, \qquad \text{.........................(4)}$$

and if we introduce two pairs of coordinated values p_1, η_1, and p_2, η_2:

$$n+1 = \frac{100}{p_1} - \frac{100\theta}{\log \eta_1} = \frac{100}{p_2} - \frac{100\theta}{\log \eta_2}. \qquad \text{..................(5)}$$

By elimination we find the value of θ:

$$\theta = \left(\frac{1}{p_1} - \frac{1}{p_2}\right) : \left(\frac{1}{\log \eta_1} - \frac{1}{\log \eta_2}\right), \qquad \text{..................(6)}$$

and by introducing the value thus found into either of the equations (5) the value of n. It is not very clear why Arrhenius suggests that n should be found by trial, as the strict procedure is certainly more expeditious as well as more elegant.

The formula (3), when applied to Miss Chick's viscosity figures, fits with remarkable accuracy, and the values of θ and n are constants over the whole range with slight exceptions. The agreement is least satisfactory for sodium caseinogenate, which shows the highest viscosity for a given weight of protein, and Arrhenius ascribes the latter to dissociation.

In order to get away from the analogy with hydrated salts—the cogency of which the author must confess himself unable to admit—and from dissociation, it seems highly desirable to test Arrhenius's formula by applying it to sols in organic solvents, such as india rubber and nitrocellulose sols. Although dissociation is certainly absent in both cases, these colloids produce, for the same weight of substance, rises in viscosity which are enormous compared even with sodium caseinogenate. They may also be expected to be highly solvated or associated, as the criterion from which Arrhenius anticipates a high degree of hydratation in the protein sols applies even more emphatically: viz. they swell enormously in their respective solvents.

The data to be used below are taken, in the case of rubber, from a paper by Schidrowitz and Goldsbrough [1909], and in that of nitrocellulose from a very complete investigation by Frank Baker [1913]. The first column shows the concentration as stated by the authors, viz. g. per 100 cc. of solution. As Arrhenius's formula contains the concentration by weight, the second column shows p, the *weight* of substance in 100 *g. of solution.* These values are exact for the nitrocellulose sols, as the densities of the sols are given by the author; for the rubber sols the densities are not available and the value of p is therefore approximate only, but in view of the low concentrations the possible error is certainly slight.

From these values of p and the observed viscosities (stated in all cases as relative viscosities, that of the solvent being taken as unity) the constants θ and n have been calculated by solving the equations (5) and (6). This involves the assumption that the method used for calculating the molecular concentration of proteins in water is equally applicable to rubber in benzene, and nitrocellulose in acetone or ether-alcohol—an assumption which is no doubt sufficiently correct, although the molecules of solute are smaller and those of solvent larger, than the molecules of protein and water respectively.

As a first example we may take a typical rubber sol (hard Para in benzene):

	g. in 100 cc.	p	η	$\log \eta$
(1)	0·25	0·281	2·5	0·39794
(2)	0·50	0·562	6·8	0·83251
(3)	1·00	1·124	32·3	1·50920

From these three sets of values we can form three pairs of equations and can thus calculate θ and n in three different ways. We thus obtain the following values:

From (1) and (2) $\theta = 1{\cdot}354$ $n = 14{\cdot}4$,
From (1) and (3) $\theta = 1{\cdot}441$ $n = -7{\cdot}5$,
From (2) and (3) $\theta = 1{\cdot}652$ $n = -21{\cdot}5$.

These values are so divergent that the formula cannot be said to apply at all. More striking even is the negative sign of n when the higher viscosities are taken into account. The mere attempt to define a *negative solvatation factor* is sufficient to demonstrate the physical impossibility of these results.

For solutions whose viscosities, for the same amount of solute, are still higher than in the preceding example, the factor n becomes negative for the whole range, while at the same time the general agreement is much improved.

As example we may take nitrocellulose in acetone (the first three sets of values in F. Baker's Table II):

	g. per 100 cc.	p	η	log η
(1)	0·2212	0·2802	3·87	0·58912
(2)	0·5261	0·6647	18·73	1·27254
(3)	0·7922	0·9977	55·87	1·74720

If we again calculate the three constants from all three pairs of equations we find:

From (1) and (2) $\theta = 2{\cdot}266$ $n = -28{\cdot}7$,
From (1) and (3) $\theta = 2{\cdot}282$ $n = -31{\cdot}4$,
From (2) and (3) $\theta = 2{\cdot}350$ $n = -35{\cdot}2$.

Here the constancy of θ and n, although far from perfect, is not unreasonably bad, especially if we consider that the assumption of a constant solvatation factor is not based on any known facts and is not inherently probable. The factor, however, is always negative and is thus devoid of any physical meaning. Nevertheless the values calculated from (2) and (3) may be used for calculating the next higher viscosity with reasonable accuracy.

If we take a further example showing still higher viscosity the results become much more erratic. The data are those for nitrocellulose in ether-alcohol (the first three values in Baker's Table XX).

	g. per 100 cc.	p	η	log η
(1)	0·3284	0·4342	8·49	0·92880
(2)	0·5977	0·7882	26·33	1·42049
(3)	0·9230	1·214	114·50	2·05866

The constants obtained are:

From (1) and (2) $\theta = 2{\cdot}75$ $n = -66{\cdot}6$,
From (1) and (3) $\theta = 2{\cdot}50$ $n = -39{\cdot}1$,
From (2) and (3) $\theta = 2{\cdot}08$ $n = -19{\cdot}7$.

Any discussion of these results is of course impossible, but the negative solvatation factors are striking.

A fourth example, in which the rise in viscosity is much more moderate, although still higher than with any protein, may be of interest. The data again refer to india rubber in benzene:

	g. in 100 cc.	p	η	log η
(1)	0·25	0·281	1·6	0·20412
(2)	0·50	0·562	2·9	0·46240
(3)	1·00	1·124	16·6	1·22011

The two constants are:

From (1) and (2)	$\theta = 0{\cdot}644$	$n = 37{\cdot}6$,
From (1) and (3)	$\theta = 0{\cdot}654$	$n = 34{\cdot}4$,
From (2) and (3)	$\theta = 0{\cdot}666$	$n = 33{\cdot}6$.

In this case the constancy of both factors is fairly satisfactory and the value of n is positive throughout. Even if it were possible, which of course it is not, to disregard the negative factors found in the previous examples, the question would still have to be answered why a solvatation factor of 34 should be possible with one colloid, whereas a factor of 8·6 should be entirely incredible in another case.

Since the formula given by Arrhenius is apparently purely empirical and is not, as far as the author is aware, deduced from any physical considerations, a discussion is possible (and called for) only on purely mathematical grounds. The logarithm of the viscosity is in the formula equated with a product, one factor of which is a constant, θ, while the other, considered simply analytically, is a function of p and of a variable parameter, n. As the $\log \eta$ curves are very simple curves of a type strongly resembling in general habitus the curves of the family

$$y = \frac{100p}{100 - (n+1)p}$$

where n, as already explained, is a variable parameter, there is at least a strong probability that by a suitable choice of n a curve can be found which will coincide with a given $\log \eta$ curve over a considerable length, within the limits of experimental error, provided both are drawn to suitable scales. The latter condition of course finds its analytical expression in the proportionality factor θ. Although the factor n is introduced with a physical meaning, we have seen that negative factors, which have no possible physical meaning, result from the application of the formula to many cases, and such negative factors occasionally achieve the desired result, i.e. more or less close interpolation or approximation to experimental results.

The formula therefore seems to be a purely analytical device for representing a viscosity, or rather $\log \eta$, curve, the approximation being achieved by a factor n which in some cases is devoid of any physical interpretation. It is therefore certainly an unwarranted assumption that values of n, which are physically possible and happen to agree with a priori assumptions such as the very dubious parallel between hydrated salts and colloids which "swell strongly in water," necessarily have the physical meaning ascribed to them, and that the higher values calculated with the aid of the author's formula are therefore necessarily incorrect.

It is only fair to add, in conclusion, that a difficulty also arises when an attempt is made to apply the author's viscosity formula to the examples treated above, or to highly emulsoid sols generally, a difficulty however which does not amount to a physical impossibility or even improbability. If the association factor is calculated say for the 0·5 % concentration of the first example ($\eta = 6{\cdot}8$) we find that about 62 % of the total volume of solvent is already associated with the solute. A 1 % sol would therefore be impossible, if the association factor were constant. There is certainly nothing inherently improbable about decreasing association with increasing concentration, and it is not difficult—as the author has found—to construct empirical functions connecting concentration and solvatation. As this is again a purely mathematical exercise affording no insight into the mechanism of association, the matter is more profitably left open until a rational solution of the whole problem, which is by no means hopeless, can be accomplished.

SUMMARY.

By applying his logarithmic formula to the viscosities of protein sols as determined by Miss Chick, Arrhenius obtains hydratation factors ranging from 0·7 to 2·2. Since these figures are of the same order of magnitude as those found for hydrates of salts in solution, Arrhenius considers that they represent the actual conditions and that for the same reason the much higher factors calculated by applying Hatschek's formula are impossible. In the present paper Arrhenius's formula is applied to sols in organic solvents and it is shown that in several instances negative association factors result, which of course have no physical meaning. The conclusion is therefore drawn that Arrhenius's formula is a purely analytical interpolation formula and that the association factors calculated from it need not, even when they happen to be positive and therefore physically possible, have the physical meaning which he ascribes to them.

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XXIX. SOME EXPERIMENTS ON FACTORS CONCERNED IN THE FORMATION OF THROMBIN.

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The experiments described in this paper were undertaken as part of an investigation into the causes of the toxicity acquired by serum from normal animals, when treated in certain ways. These toxic properties have been supposed by a number of investigators to be related to the phenomena of the anaphylactic reaction, and the name "anaphylatoxin" or "serotoxin" has been applied to the hypothetical toxic constituent. Some have supposed that the toxic substance is a protein cleavage-product; others that the toxicity is due to some undefined alteration in the physical properties of the colloidal system. We may remark that it is not as yet clear, that the toxic action produced by the various treatments, to which the serum has been exposed by different workers, is in all cases the same. Our attention was first directed to the toxic action, which fresh guinea-pig's serum very regularly acquires when shaken with about twice its volume of chloroform, as described in a series of papers by Jobling [1914] and his co-workers. We were struck by the conspicuous part played, in the toxic action of such a serum, by massive intravascular clotting. The occurrence of clots in the heart is mentioned, indeed, incidentally by these authors, but is evidently regarded by them as a subsidiary feature of the action. It may be so. We decided, however, to begin our proposed analysis of the action by examining this aspect of the effect, and were thus led to make a series of experiments on the phenomena of blood-clotting in vitro, and the effect, on the factors concerned, of treating plasma with chloroform, along similar lines to those which have been found to produce toxicity of sera.

A few preliminary experiments were made with mammalian serum and plasma. The investigation was complicated, however, by the necessity of using a calcium precipitant to secure a plasma which did not coagulate spontaneously. We turned, therefore, to the plasma of the fowl, which several investigators have used on account of its stability when carefully prepared. The examination of the changes produced in it by treatment with chloroform necessitated the preparation of a series of the individual clotting-factors in as nearly pure a condition, in a physiological sense, as was practicable; and we were thereby at once involved in a consideration of different theories of blood-coagulation. For the mere definition of the factors concerned in normal clotting, and of the criteria of physiological purity to be applied to them, involves at least a provisional acceptance of one of the theories of their nature and function.

Our purpose in this preliminary paper, therefore, is to describe the methods which we have employed in preparing test-solutions of the clotting factors, and the information which we have obtained, by their use, concerning the processes occurring in a plasma treated with chloroform. The results so obtained seem to us incidentally to give very clear evidence against one recently propounded conception of the clotting-process. Later, when external conditions are more favourable to the steady pursuit of such an investigation, we hope to apply these methods and results to the study of induced toxicity of sera, and of the problem of anaphylaxis.

THEORIES OF BLOOD COAGULATION.

We are not immediately concerned with the mode of origin, or the precise nature of the interaction between the clotting-factors. So many reviews of the large literature of the subject have appeared, that we need only indicate briefly the main points of the two conceptions between which we attempt a decision. The principal current conception was present, in its essential features, in the theory finally put forward by Alexander Schmidt [1892, 1895], at the conclusion of his protracted and classical investigation; though in Schmidt's statement it was obscured by other conceptions which later work has failed to uphold. The theory, in its present relatively clear form, was put forward independently by Morawitz [1904] and by Fuld [1903], and numerous other investigators have contributed evidence as to the mode of origin and interaction of the factors concerned. The theory involves the interaction of three original factors, in addition to calcium ions, and the variety of the nomenclature used by different authors is somewhat bewildering. The factors are (1) fibrinogen, the soluble globulin of plasma which is converted into insoluble fibrin by the action of "thrombin" (fibrin ferment);

(2) an inactive precursor of thrombin, present in circulating blood and in stable plasma, which has been variously called plasmozyme, serozyme, thrombogen, and for which (following Mellanby [1908] and other recent writers) we use Schmidt's term "prothrombin," though this involves some restriction of Schmidt's application of the term; and (3) a substance obtainable from cellular tissues, or from blood-platelets, which in the presence of calcium ions brings about the conversion of the inactive "prothrombin" into the active thrombin. This substance was recognised by Schmidt, and by him called "zymoplastic substance." It corresponds to the "*A*-Fibrinogen" of Wooldridge, and has been variously termed thrombokinase (Morawitz), cytozyme [Fuld and Spiro, 1904], and, more recently, "thromboplastin" [Howell, 1910]. We use here, again following Mellanby, Morawitz's term "thrombokinase," or more briefly "kinase," for this factor.

An alternative conception, put forward in recent years by Howell [1911], and elaborated in a series of communications from his laboratory, differs from the foregoing in the importance attributed to "antithrombin." The presence in plasma or serum of factors inhibiting the coagulant effect of thrombin on fibrinogen had been recognised by a number of earlier investigators, and their action collectively attributed to "antithrombin." Howell supposes that prothrombin is converted into thrombin simply by the action of calcium, and that this action is prevented in circulating blood, or in stable avian or reptilian plasma, by the fact that prothrombin is associated with antithrombin. The rôle of the kinase (thromboplastin), according to Howell's view, is to dissociate this complex, by combining with the antithrombin, freeing the prothrombin to the action of the calcium ions.

It is clear that an acceptance of one or other of these theories is implicit in any definition of the clotting-factors, when an attempt is made to prepare them separately. Howell, for example, regards a preparation as pure prothrombin, in respect of its activity, if it fails to clot fibrinogen when added in the absence of calcium ions, but clots it promptly if an ionised calcium salt be added as well. He would regard a prothrombin, which fails to produce clotting under such conditions, and needs the further addition of kinase for its activation, as contaminated with antithrombin. Adherents of what we may, for brevity's sake, term the Morawitz theory would conclude, on the other hand, that the activation of Howell's "prothrombin" by calcium ions alone was clear proof of its contamination with kinase; it might even be kinase only, without any prothrombin, if the fibrinogen used had not been carefully freed from the prothrombin which is readily precipitated with it.

PREPARATION AND TESTING OF REAGENTS.

1. *Fibrinogen.* The only difficulty in preparing an adequate fibrinogen solution is to get it absolutely free from prothrombin. Prothrombin is either itself a euglobulin, or is closely associated with one. Mellanby, indeed, appears to regard fibrinogen and prothrombin as so closely associated, that he frankly abandons the possibility of separating them, and uses as "fibrinogen" a preparation from which prothrombin can be separated by clotting the fibrinogen with thrombin.

This practice has the disadvantage that the decision, whether a given solution contains thrombin or kinase, is frequently a matter of indirect inference; and when both are present in the preparation being examined, the presence of adventitious prothrombin in the reagent fibrinogen makes the interpretation of results excessively complicated and difficult.

To obtain a fibrinogen free from this defect we used a device indicated by Bordet and Delage [1912]. These authors showed that an oxalated plasma, exposed to the adsorbing influence of precipitated barium sulphate, becomes indefinitely stable on recalcification, and will no longer clot when kinase is added in any amount, but yields a good clot with thrombin.

A plasma so treated ought, therefore, to yield a fibrinogen free from prothrombin. We find that it does so. A fair amount of the fibrinogen is apparently lost on the adsorbent barium sulphate; but enough remains to make easy the preparation of a strong fibrinogen solution. As a precipitant of fibrinogen we have used an equal volume of saturated sodium chloride, following Hammarsten [1879]. Mellanby suggests that Schmidt's earlier method of precipitating by dilution and acidification gives a less denaturated product. Our experience with oxalated rabbit-plasmata is quite contrary to this. We found that dilution and acidulation, even with the greatest care to attain the optimum reaction, produced in such plasmata a scanty precipitate, in an inconveniently large volume of fluid. This precipitate, collected by the centrifuge, was seldom perfectly soluble in neutral saline, and became less so if it were taken up in dilute sodium carbonate, and reprecipitated by dilution and acidulation. We therefore adopted the following routine procedure, which is similar to that employed by Howell, except that the plasma was freed completely from prothrombin before precipitation.

Rabbits were etherised and bled from the carotid artery, through a paraffined cannula, into large centrifuge tubes. 10 cc. of a watery solution containing 1 % potassium oxalate with 0·9 % sodium chloride were put into each tube as a preliminary. If the volume of blood obtained exceeded 90 cc., an extra amount of the oxalate solution was added as soon as the 100 cc. mark was attained. The oxalated blood was cooled and thoroughly centrifuged. The plasma was carefully pipetted off, rejecting the layer immediately in contact with the corpuscles and platelets.

A standard suspension of barium sulphate was prepared as follows. 31·5 g. of barium chloride were precipitated with a calculated slight excess of sodium sulphate, and washed repeatedly with 1 % sodium chloride solution by decantation, until the washings were free from sulphate. The total volume was then made up with 1 % sodium chloride to 200 cc. To every four volumes of oxalated plasma were added three volumes of the well-shaken suspension of barium sulphate.

The mixture of plasma and barium sulphate was well stirred, and allowed to stand for some hours—usually over night. It was then centrifuged clear, and the plasma poured off from the heavy barium sulphate. To each volume of the now diluted plasma was added an equal volume of a saturated solution of pure sodium chloride. Precipitation occurs rather slowly. As soon as separation was complete—i.e. usually after about an hour—the precipitate was separated with the help of the centrifuge, dissolved in 2 % sodium chloride, and again precipitated with an equal volume of saturated sodium chloride. The precipitate was collected again and dissolved in a convenient volume of 2 % sodium chloride. A trace of sodium hydrate was added if necessary to assist solution, and a measured equivalent quantity of standard hydrochloric acid then added to neutralise. The solution was centrifuged clear from any flakes of denaturated material, if such were present, and finally diluted with water to a content of 1 % sodium chloride. Such a solution is, apparently, indefinitely stable if kept cold. It yields a hard clot in a few minutes if a drop of active thrombin be added to a few drops of the fluid. The following represents the usual test and its result:

Fibrinogen solution 5 drops + 1 % sodium chloride 10 drops + thrombin solution 1 drop. Put into bath at 37°.—Clotted firmly in 2½ minutes.

[In recording subsequent experiments we adopt the convention of using only the name of a clotting factor to indicate the stock solution of it here described. The figure following it indicates the number of drops added. Saline = 1 % pure sodium chloride. Calcium = 1 % calcium chloride. All determinations were done in a water-bath at 37°.]

On the other hand, neither of the other clotting-factors can be made to clot such fibrinogen solutions when added separately. With either prothrombin and calcium, or kinase and calcium, using these terms as defined below, no trace of clotting could be observed, however long the incubation was continued.

2. *Prothrombin.* It is evident that the definition of "prothrombin" involves adhesion to one or the other of the two theories mentioned above. Since one of our objects was to discover additional facts which would not, as are so many of those hitherto available, be explicable on either theory, we were obliged to make our definition in a sense experimental. Previous workers have obtained prothrombin preparations, which either failed altogether to clot fibrinogen with calcium only, or did so extremely slowly, and had their action enormously accelerated by kinase. On the Morawitz theory, such preparations are prothrombin, either pure, or contaminated by traces of thrombin. On Howell's view, they are prothrombin which cannot become thrombin in the presence of calcium, on account of associated antithrombin. If, therefore, we could prepare a prothrombin which showed no sign of activation with calcium alone, which rapidly yielded thrombin when kinase in addition was added, and which contained no demonstrable antithrombin, we should have good reason for not accepting Howell's view. There would remain, in regard to that view, only the duty of showing how Howell's evidence in its favour could be explained along other lines. We are convinced that we have on numerous occasions obtained such preparations of prothrombin, practically free alike from thrombin and from antithrombin, not activated by calcium alone, but readily by kinase in addition. One method of obtaining such is the treatment of fowl's plasma with chloroform under appropriate conditions, which we discuss in a later section. Such preparations, however, are very unstable, for reasons which we there discuss. Moreover the conditions governing their production are so complex, that there is an element of uncertainty in the method, as we shall also show later.

Mellanby's method of preparing prothrombin from fowl's plasma is admirable as a practical process. From the controversial point of view it has two defects. It is difficult to add just so much thrombin that all the fibrinogen is removed from the solution containing it and prothrombin, and no excess of thrombin left. One easily obtains prothrombin with some fibrinogen, still capable of yielding a weak clot when kinase and calcium are added; and one easily obtains prothrombin with so little thrombin, that a quantity of the preparation, which clots fibrinogen in a few seconds after treatment with kinase and calcium, will clot it only after half an hour or more, when added with calcium alone. But this presence of a trace of thrombin gives to the action of the kinase the appearance of a mere acceleration. And this is important, as being capable of reconciliation with Howell's ideas; since Mellanby's method unmodified gives no guarantee against the presence of some antithrombin, albeit in traces which would not seem to us significant. By a slight modification, however, prothrombin, free alike from perceptible traces of thrombin or antithrombin, can be obtained.

In a fowl anaesthetised with ether the main artery of the leg is exposed, isolated, tied, and clamped proximally to the ligature with bull-dog forceps. The surface is washed carefully with oxalated saline, a cut with clean scissors made between ligature and bull-dog clamp, and the interior and cut surface washed through a capillary pipette with the oxalated saline. A clean glass cannula is then tied in and the blood allowed to flow straight into a large centrifuge tube. In this the blood is rapidly cooled to nearly 0° C., and is then centrifuged for about 15 minutes at 4000 revolutions per minute with a mean radius of 20 cm. The clear plasma is pipetted off. If stored cold, such a plasma seems to remain indefinitely stable. It does not clot at 37° C. within any reasonably limited time of observation. If diluted with two or three volumes of water, it usually clots after some hours.

A mixture of fibrinogen and prothrombin can be precipitated from such a plasma, as Mellanby describes, by diluting with twenty volumes of water and bringing the reaction to the isoelectric point of the globulins by adding acid. The association of fibrinogen and prothrombin in this precipitate is regarded by Mellanby as indicating their association as a preformed complex in the plasma. The force of this suggestion seems to us to be weakened by certain observations of our own. We have already described how fibrinogen can be prepared free from prothrombin by adsorbing the latter with barium sulphate. Mellanby showed that prothrombin was left in solution when a fibrinogen-prothrombin mixture was clotted with thrombin. We have now to add evidence that the two can be separated by fractional precipitation, brought about by careful reduction of electrolyte content. It seems to us, therefore, that the regularity with which fibrinogen and prothrombin occur together, in precipitates obtained by diluting and neutralising plasma, merely signifies that prothrombin has the solubilities of a euglobulin, like fibrinogen. It appears, however, to be soluble in a weaker solution of pure sodium chloride than is required to dissolve fibrinogen. The details of an actual preparation will explain the point.

50 cc. of clear, fresh fowl's plasma were diluted with 1 litre of distilled

water. One-tenth normal acetic acid was then added till maximum precipitation was obtained, 15·75 cc. being required. The precipitate was collected by the centrifuge, rubbed up with distilled water and again separated. It was then dissolved in 18 cc. of 1 % pure sodium chloride solution. The solution still showed a trace of the yellow colour of the original plasma. 11 cc. of it were poured into a glass vessel and warmed to 37° in a water-bath. A stock solution of thrombin was added, one drop every half-hour, till a weak clot began to form. The solution was then removed and allowed to stand at laboratory temperature over night. Next morning the now solid clot was broken up, and the serum removed. The object was to remove most of the fibrinogen without leaving any excess of thrombin in the serum. This was apparently attained, since five drops of the serum failed to clot five drops of fibrinogen solution in several hours. The serum, however, still contained some fibrinogen, since a sample yielded a soft clot in a few minutes on adding kinase and calcium. It also contained a remnant of "antithrombin," as we shall show, and was, therefore, further treated as follows. It was first dialysed for 4 hours against distilled water in a collodion tube. A fairly copious precipitate formed, and a specimen of the supernatant fluid on testing was found to be relatively weak in prothrombin, and to possess a weak, but distinct antithrombic action. The contents of the dialysing tube were therefore poured out and spun in the centrifuge. The supernatant fluid was then poured off, being retained for a test of its antithrombic action, and the precipitate was dissolved in 10 cc. of 1 % sodium chloride. On dilution with 10 cc. of distilled water no precipitation occurred; but on adding a further 10 cc., reducing the sodium chloride content to 0·33 %, a flocculent precipitate was formed. This contained practically all the fibrinogen; for the supernatant fluid, separated clear by the centrifuge, no longer gave any trace of clot with kinase and calcium, though it contained a powerful prothrombin. This fluid was, indeed, such a powerful prothrombin that it was diluted with twice its volume of 1 % sodium chloride, for routine use. Experimental details may be given of the tests made of its properties, since the results seem to us to constitute a very clear refutation of Howell's theory. On this theory, the solution, having all the properties of prothrombin in a high degree, should (*a*) yield thrombin on treating with calcium ions alone, or (*b*) contain at least a definitely recognisable amount of antithrombin. We find no trace of either action.

EXPERIMENT 89 (*b*).

Test of solution (*S*) *for thrombin.*

(*a*) Preformed.
Fibrinogen 5 + saline 7 + *S* 5.—No clot in 1 hour.

(*b*) Formed in presence of calcium ions.
Fibrinogen 5 + saline 7 + calcium 1 + *S* 5.—No clot in 1 hour.

Test for prothrombin.

S 5 + calcium 1 + kinase 2—incubated together 10 minutes; then added: Fibrinogen 5 + saline 7.—Hard clot in 30 seconds.

Test for "antithrombin." In using, for convenience, the term "antithrombin," we have no intention of committing ourselves to the assumption that plasma or serum contains a definite substance, having a specific affinity for thrombin, and a neutralising effect on its action. What is definitely known is, that plasma and serum contain some substance or substances which hinder and delay clotting. Some may, and probably do, act by becoming physically associated with the thrombin; others may hinder the gelation of the fibrin. Howell seems to assume that the substance, which delays the action of formed thrombin, is the same as that which, on his theory, hinders the combination of prothrombin with calcium to form thrombin. The reason for this assumption is not clear to us, but a good deal of Howell's evidence is directed to showing that kinase weakens the effect of the factor delaying the action of formed thrombin. We are, therefore, entitled to suppose that the presence of this factor in a prothrombin, which is not activated by calcium ions alone, is essential to his argument. This factor has been shown by Howell, among others, to be stable at 60°; and since our thrombin contains traces of kinase, all solutions containing prothrombin had to be freed from the latter by heating to this temperature before their antithrombic value could be fairly tested. In comparative tests, like the following, the preparations were heated side by side, in the same water-bath, being immersed and removed together. The thrombin solution was diluted so that one drop, added to the standard fibrinogen dilution, caused clotting in about 10 minutes. In addition to the reagents already specified, we prepared heated fowl plasma, and heated solution of prothrombin (*S*), the heating being carried out by immersion in a water-bath at 60° for 20 minutes. The following are the tests and their results:

Experiment 89 (*b*) (*continued*).

1. (Control.) Fibrinogen 5 + saline 12 + diluted thrombin 1.—Clotted in 11 minutes.

2. Fibrinogen 5 + heated prothrombin 7 + saline 5 + thrombin 1.—Clotted in 11 minutes.

3. Fibrinogen 5 + heated plasma 7 + saline 5 + diluted thrombin 1.—No clot in 3 hours. Found clotted next morning—16 hours.

It is quite clear, then, that the prothrombin solutions contain no recognisable fraction of the substance inhibiting thrombin action, which can be demonstrated in the original plasma. When the original fibrinogen + prothrombin precipitate, obtained according to Mellanby's directions, was purified by redissolving, and then again precipitating by dialysis, the supernatant fluid from the dialyser was found to contain a trace of the antithrombin, as the following results show. The solutions were heated together to 60° as before, but a thrombin of twice the strength was used in this experiment.

1. (Control.) Fibrinogen 5 + saline 12 + thrombin 1.—Clotted hard in 5½ minutes.

2. Fibrinogen 5 + saline 5 + heated prothrombin 7 + thrombin 1.—Clotted hard in 5½ minutes.

3. Fibrinogen 5 + saline 5 + heated supernatant fluid from dialyser 7 + thrombin 1.—This also showed signs of clotting after 5½ minutes in the thermostat, but the clot was soft, and was not properly hard even in 1 hour.

4. Fibrinogen 5 + saline 5 + heated plasma 7 + thrombin 1.—An extremely soft clot began to form after 11 minutes, and was still soft after 1 hour.

We have found, in experiments on the effect of varying quantities of antithrombin, that a delay of the firm setting of the clot appears with amounts which no longer clearly delay its first appearance. We feel entitled, therefore, to conclude that small quantities of the antithrombin of plasma adhere to the precipitate of fibrinogen and prothrombin, obtained from the plasma by dilution and adjustment of reaction. These, however, can be removed by reprecipitating the mixed euglobulins by dialysis. There is no indication of the formation of thrombin from the prothrombin during the process, nor on the subsequent addition of calcium salts. On the subsequent addition of kinase, thrombin is formed, and, if fibrinogen is present, this is clotted. A further series of tests may be quoted, to illustrate this last point, together with the other properties of a preparation of prothrombin made according to Mellanby's directions, without further purification. This specimen, when freshly prepared, contained some thrombin, and probably, from previous experience, traces of antithrombin. After keeping in the cold for a few days, no thrombin or antithrombin could be detected in it. Physiologically it was now a pure and powerful prothrombin, though its activity in this respect had also undergone slight decay.

EXPERIMENT 88.

1. *Test for prothrombin.*

Solution 5 + calcium 1 + kinase 2.—Incubated 10 minutes.

Then added fibrinogen 5 + saline 7.—Clotted in 1½ minutes.

2. *Test for preformed thrombin.*

Fibrinogen 5 + saline 10 + solution 5.—No clot in 3 hours.

3. *Test for formation of thrombin with calcium ions, but no kinase.*

Fibrinogen 5 + saline 7 + solution 5 + calcium 1.—No clot in 1 hour.

Then added: kinase 2—clotting began in 12 minutes, and was complete in 17 minutes.

4. *Test for antithrombin,* in solution heated to 60° for 8 minutes.

As a control we used, in this case, a fowl-plasma which had lost some of its antithrombin value by keeping. It was heated to 60° for 20 minutes.

Control 1. Fibrinogen 5 + saline 12 + diluted thrombin 1.—Clotted hard in 18 minutes.

Control 2. Fibrinogen 5 + heated fowl-plasma 7 + saline 5 + diluted thrombin 1.—Weak, soft clot in 1½ hours.

Test. Fibrinogen 5 + heated solution 7 + saline 5 + diluted thrombin 1. —Clotted hard in 18 minutes.

Here, then, we have again a prothrombin, which contains no demonstrable antithrombin. It is activated by kinase in the presence of calcium to a powerful thrombin, but even prolonged incubation with calcium alone fails to produce any thrombin from it; the only effect of such long incubation, with calcium and fibrinogen, is an apparent delay in the subsequent activating effect of kinase.

3. *Thrombin.* We have not prepared a pure thrombin solution. We required thrombin mainly as a test for the continued presence of fibrinogen in a solution, which has failed to clot with other reagents, and for testing the antithrombic effect of various preparations. Provided the latter contained

no prothrombin, which was excluded by previous heating, the existence in our thrombin of a trace of kinase, which it undoubtedly contained, was immaterial. It was prepared by whipping the fibrin from rabbit's blood, as shed from the artery; washing the fibrin till white by kneading with water; and then extracting with 8 % sodium chloride, according to Howell's modification of Gamgee's method. The thrombin so obtained was, as a rule, unnecessarily powerful for general use. It was usually kept in the strong solution, and portions diluted as required. Diluted with an equal volume of water, it gave a solution of which one drop caused firm clotting of our standard mixture of 5 drops of fibrinogen + 10 drops of saline in 2 or 3 minutes. For detecting small amounts of antithrombin it was diluted further, so that one drop clotted such a mixture in 10 to 20 minutes. After heating to 60° the strong solution showed no thrombic action, failing to clot pure fibrinogen solutions in any period. A weak activating (kinasic) effect on prothrombin in the presence of calcium remained after such treatment.

4. *Thrombokinase.* As a source of kinase, following Mellanby's recommendation, we used the fowl's testis, stripping off the capsule with its blood-vessels. The pulp was allowed to stand at room temperature for a day, and then treated by Howell's method—i.e. it was ground up with sand, dried, and then rubbed up with glycerol. The mixture was centrifuged and the turbid supernatant fluid, unfiltered, was diluted as required with two volumes of distilled water. Such dilutions appeared to keep well for a week or two, and then suddenly to lose their effect. The strong glycerol extract seemed to be indefinitely stable.

THE EFFECT OF CHLOROFORM ON PLASMA, ETC.

As stated in the introduction, our ultimate object is a closer analysis of the effect of extraction with chloroform, and other forms of treatment, in imparting toxicity to sera. A more immediate interest was given to the matter, from our present point of view, by the discovery that shaking with chloroform, which has been shown by Jobling and his associates to destroy antitrypsin in serum, very rapidly and completely destroys the antithrombic action of serum or plasma. Since we first observed this effect, a paper has appeared by Minot [1915], in which he shows that shaking oxalated mammalian plasma with a little chloroform will frequently induce clotting. He, rightly in our opinion, attributes this to the effect of some preformed thrombin, which is able to act when the antithrombin has been destroyed.

He further reaches the conclusion that chloroform does not convert prothrombin into thrombin as Howell [1910] had originally supposed. Minot's meaning seems to be that the mere removal of antithrombin, in the absence of calcium, does not activate Howell's preparation of prothrombin, of which we consider the properties in another section.

Our own first experiments with fowl's plasma gave results which, at first sight, seemed to be strongly in favour of Howell's theory. On shaking a small volume of fowl's plasma with twice its volume of chloroform, keeping the mixture warm in a water-bath at 37°, we found that, after about 10 minutes, the emulsion suddenly acquired a semi-solid consistency, as though clotting had occurred. The now permanent emulsion was kept at 37° for about an hour, and then removed to centrifuge tubes, and spun rapidly for about 15 minutes. The clear, supernatant watery layer was pipetted off and tested. It was found to possess a very powerful thrombin action. After dilution to five times its volume, one drop clotted the standard fibrinogen mixture in 2 minutes. After heating to 60° the preparation was found to be devoid of antithrombic action. This observation lends itself readily to interpretation in accordance with Howell's views. Fowl's plasma is known to contain much prothrombin, and a good deal of antithrombin. According to Howell, it is this latter which prevents the prothrombin from becoming thrombin by union with calcium ions. We have removed the antithrombin by chloroform treatment, and thrombin is formed. To that extent, therefore, the expectation is fulfilled. A few further experiments, however, sufficed to dispel this plausible interpretation of the effect.

In the first place, the fluid obtained by chloroform treatment of the fowl-plasma, readily produced clotting of many times its own volume of the plasma from which it was produced, or of any other stable fowl-plasma. For example, the following mixtures were placed in the bath at 37°, with results as indicated:

EXPERIMENTS 48 AND 49.

1. Fowl's plasma—5 drops.

Saline 1 drop.—No clot in 24 hours.

2. Fowl's plasma—5 drops.

Chloroform-treated fowl's plasma (as above)—1 drop.—Clotted firmly in 1½ minutes.

3. Fowl's plasma—10 drops.

Chloroform-treated fowl's plasma—1 drop.—Clotted firmly in 2½ minutes.

If the activation of the plasma were to be explained as the result of removal of antithrombin, the result in tube No. 3 would oblige us to conclude, that the loss of not more than 1/11 of the total antithrombin present in a sample of the plasma allows the formation of sufficient thrombin to clot the whole in a few minutes. And this conclusion would be wholly at variance with all other experience of the neutralising effect of antithrombin on the action of thrombin. If we are dealing with a sample of antithrombin, of which eleven drops so delay the action of a given dose of thrombin that the clotting-time is lengthened from, say, 10 minutes to 5 hours, then we shall find that ten drops of the same antithrombin prolong the clotting-time to 4½ or 4¾ hours, perhaps; but that clotting should occur in 2½ minutes with ten drops, and be altogether inhibited by eleven drops of an antithrombic preparation from plasma or serum, is an impossible result.

It might possibly be urged that this argument only applies to the free antithrombic constituent of serum or plasma, which survives heating for some minutes to 60°; and that the possibility remains of prothrombin being associated in plasma with some other thermolabile inhibitory factor, which is similarly liable to the destructive action of chloroform. Such a suggestion, however, has no direct bearing on either the evidence or the theory which we are discussing. The only factor, of whose destruction by chloroform we have direct evidence, is thermostable; and it is an essential feature of Howell's view, if we rightly understand it, that it postulates an association between prothrombin and this thermostable antithrombin, which is not readily destroyed at 60°; and that it attributes to the effect of this factor the inability of calcium alone to activate a prothrombin preparation. It is easy, moreover, to dispose of the suggestion by experiment. If the activation of fowl-plasma by chloroform were due to the removal of an antithrombic factor of any kind, it should clearly be possible to activate, by similar treatment, a prothrombin solution prepared from that plasma. This we have repeatedly attempted, with no hint of success. If a prothrombin, prepared from fowl's plasma according to the indications of a previous section, be shaken with chloroform, either in the cold or at 37°, the prothrombin is fairly rapidly destroyed, but no trace of thrombin appears. As long as activity of any kind remains, it is that of prothrombin—i.e. of a substance which clots fibrinogen only after treatment with calcium and kinase, and never with calcium alone.

We may conclude, then, that the activation of fowl-plasma by chloroform is due neither to the removal of antithrombin, revealing a previously neutralised thrombin, nor to direct action of the chloroform on the prothrombin. To elucidate the nature of the action we decided to examine the different layers of the plasma-chloroform emulsion, after different periods of treatment at varying temperatures, for the presence of the several known clotting-factors, using the set of reagents previously described.

It should be stated at once that the results obtained, though all pointing to the same conclusion, showed considerable variation. After shaking a specimen of stable fowl-plasma for a given time and at a given temperature with chloroform, separating the emulsion by the centrifuge, and then examining separately the clear supernatant watery layer, and the emulsion of more or less denaturated material separating with the chloroform-layer, we found it impossible to prophesy, in any given case, the nature and distribution of the clotting factors which we should find. It varied, not only with the plasma from different fowls, but with the same sample of plasma, after different periods of storage in the cold-room. The reason of this variability in the result will be sufficiently clear, when we have described the experiments which demonstrate the complexity of the processes at work. We mention it here in explanation of the fact, that we choose, for extended description, experiments in which the conditions made it possible to follow clearly the stages of the production of thrombin. No results were obtained which were inconsistent with the conception thus obtained; but many in which intermediate stages were missed, or in which, owing to changes which will later become clear, the process was incomplete.

The procedure adopted was, in its general features, identical in all cases. To one volume of the plasma we added two volumes of chloroform. It is quite probable that a much smaller volume would have effected the changes which we describe; but to experiment with different proportions would have made the number of tests required unmanageably great. After the two had been shaken together under the conditions of the particular experiment, the mixture was thoroughly centrifuged. The clear watery layer could then be poured off clean from the stiff emulsion which formed the upper portion of the chloroform-layer. The watery layer was, if necessary, freed from traces of dissolved chloroform by blowing air over it. The chloroform-layer was then stirred, and the free, fluid chloroform poured away. The curdy emulsion left was then rubbed up with a volume of 1 % sodium chloride solution equal to twice the volume of plasma taken and over this a vigorous stream of air was blown until all chloroform was removed. Part of the material passed into solution during this process; part, in most cases, was rendered permanently insoluble by the denaturating effect of the chloroform.

We had, then, for examination the supernatant watery layer, and the saline extract of the chloroform-layer. These will be referred to as "watery layer" and "chloroform-layer" respectively.

A preliminary, incomplete experiment may first be quoted.

EXPERIMENT 52.

A sample of fowl-plasma was shaken with chloroform till a permanent emulsion was formed, and this was left over night at laboratory temperature. Next morning the mixture was centrifuged and the two layers examined. The watery layer had a rather weak thrombin-action, as the following experiment shows:

Fibrinogen 5 + saline 5 + watery layer 1.—Clotted in 12 minutes.

The chloroform-layer, on the other hand, was much weaker in this respect:

Fibrinogen 5 + saline 5 + chloroform-layer 1.—Clotted in 50 minutes.

But the chloroform-layer, though having but weak thrombin action, revealed itself, on incubation, with prothrombin and calcium, as an effective kinase.

Prothrombin 5 + calcium 1 + chloroform-layer 2, incubated for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted in 4 minutes.

Here, then, was an indication that the chloroform treatment produces from the stable plasma something which functions as a kinase. In this experiment no attempt was made to study its distribution between the layers; it was merely shown that it was present in the chloroform-layer, which contained little thrombin. We shall show, by quoting other experiments, that this distribution between the layers is very variable. Several of the earliest of our complete experiments gave the impression that the kinase, after the centrifuging of the emulsion, was practically limited to the chloroform-layer. Later, however, we met with cases in which, after apparently identical treatment of a plasma, the watery layer had a very high kinasic value, while that of the chloroform-layer was hardly perceptible. We shall discuss the probable meaning of these discrepancies after illustrating them. For the present, the point of importance is that the chloroform-treatment caused the appearance of kinase in plasma, which certainly did not contain it before, since the untreated plasma was indefinitely stable even at 37°. We may add at once, also, that after treatment with chloroform at 37° for several days, kinase is the sole clotting-factor which can be discovered in either the watery layer, or in what remnant of water-soluble constituents the chloroform-layer then contains. In one such case the incubation was carried on from a Saturday afternoon to the following Monday morning. After centrifuging, the watery layer was tested as follows:

1. *Test for thrombin.*

Fibrinogen 5 + saline 10 + watery layer 4.—No trace of clotting in 1 hour.

One drop of a known thrombin was then added to the tube and produced a firm clot in a few minutes. We may conclude, then, that the fluid under examination contained no thrombin.

2. *Tests for kinase.*

Prothrombin 5 + calcium 1 + watery layer 2, incubated together for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted firmly in less than 30 seconds.

The clotting-time being so short, the test was repeated, exactly as above, using, however, the watery layer diluted, first four times, and then twenty times. The clotting times were 2 minutes and 16 minutes respectively. Clearly, then, we were left with a very powerfully kinasic solution, while all thrombin, and necessarily all prothrombin, had vanished.

This discovery, of the formation of kinase in plasma treated with chloroform, at once suggested an explanation of the conversion of prothrombin into thrombin in a plasma so treated.

It will be clear that the combination of effects, thus demonstrated as resulting from the action of chloroform, gives us another opportunity of putting to the test the rival theories with which we are here concerned. On the Morawitz-theory the formation of thrombin is obviously explicable by the action of the freshly-liberated kinase on the prothrombin, in the presence of calcium. On Howell's theory the disappearance of the antithrombin would be the decisive feature of the action, the appearance of kinase only having significance, in so far as it assisted this. It occurred to us that a clear indication, in support of one or the other of these views, might be obtained by carrying out the chloroform treatment in the absence of calcium. It seemed probable that antithrombin would still be destroyed, and kinase still formed under such conditions, though activation of prothrombin would not take place till calcium was added. If, then, we could effect a separation of the prothrombin and the newly-formed kinase by the centrifuge, the effect of adding calcium would give us a definite answer; for on Howell's theory the layer containing antithrombin-free prothrombin should form thrombin with the calcium only, while on the Morawitz theory, admixture with the kinase-containing layer should be necessary before activation occurred. All the conditions indicated have been realised in a series of experiments, and the result seems to us quite decisively against the theory of Howell, and in favour of that of Morawitz.

We may quote first an experiment on the action of chloroform on a further sample of the plasma used in Experiment 52.

EXPERIMENT 53.

To 15 cc. of plasma was added 1·5 cc. of 1 % potassium oxalate. 30 cc. of chloroform were then added, and the whole shaken mechanically at laboratory temperature for 1 hour. It was then thoroughly centrifuged and the two layers examined separately as described above.

Tests of the watery layer.

(1) *For thrombin.*

(*a*) Fibrinogen 5 + saline 10 + watery layer 5.—No clot after 2½ hours.

(*b*) Fibrinogen 5 + saline 10 + calcium 1 + watery layer 5.—No clot after 2½ hours.

Therefore no thrombin was present, and none was formed on addition of sufficient calcium chloride to remove excess of oxalate, and secure the presence of free calcium ions.

On adding two drops of kinase to each of the above tubes, at the end of 2½ hours, (*a*) showed no sign of clotting in a further hour, while (*b*) clotted in about 12 minutes—thus showing that it contained an effective amount of free calcium, as well as prothrombin.

(2) *For prothrombin.*

Watery layer 5 + calcium 1 + kinase 2—incubated together for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted in 4 minutes.

Therefore a moderate amount of prothrombin was present; and since, as shown under (1), no thrombin was formed with calcium alone, no intrinsic kinase can have been present in the watery layer.

(3) *For antithrombin.*

A weak thrombin was used, of which four drops clotted the standard fibrinogen dilution only after 10 minutes.

(*a*) Fibrinogen 5 + saline 11 + thrombin 4 + calcium 1.—Clotted in 10 minutes.

(*b*) Fibrinogen 5 + heated watery layer 8 + saline 3 + calcium 1 + thrombin 4.—Clotted hard in 14 minutes.

(c) Fibrinogen 5 + heated fowl-plasma 8 + saline 3 + calcium 1 + thrombin 4.—*Not* clotted in 100 minutes.

The trivial delay of clotting, from 10 to 14 minutes, by the heated watery layer cannot be regarded as indicating the presence of antithrombin. It is an effect such as might be produced by a slight difference in saline concentration, or slight increase of the alkalinity of the mixture. It will be noted that, under the same conditions, the heated original plasma lengthened the clotting-time to over 100 minutes—beyond which period the observation was not continued.

Tests of the chloroform layer.

1. *Tests for thrombin.*

(a) Fibrinogen 5 + saline 8 + chloroform-layer 4.—No clot in 30 minutes.

(b) Fibrinogen 5 + saline 8 + chloroform-layer 4 + calcium 1.—No clot in 30 minutes.

Therefore no thrombin was present, and none was formed in the presence of calcium.

2. *Test for kinase.*

Prothrombin 5 + calcium 1 + chloroform-layer 4, incubated together for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted in 2 minutes.

Since, therefore, this layer contained plenty of kinase, it is clear that it contained no prothrombin, since the addition of calcium alone (under 1) did not produce any thrombin from it.

We have, therefore, in this experiment, by treating oxalated fowl-plasma with chloroform, produced kinase, which, however, in the absence of calcium, could not activate the prothrombin. Fortunately for the purpose of our enquiry, the kinase, in this instance, is found, after the emulsion has been centrifuged, to be limited to the chloroform-layer. The watery layer contains an adequate prothrombin, free from clearly recognisable antithrombin. Yet this prothrombin is not activated by treatment with calcium only; kinase in addition activates it promptly. We are entitled to conclude, therefore, that the formation of thrombin, when fowl-plasma, containing its normal calcium, is shaken with chloroform, is due to the production of kinase, which secondarily activates the prothrombin. The disappearance of antithrombin is an accidental accompaniment, not an essential feature of the process.

The clean separation of the prothrombin and kinase by the centrifuge, as might be expected, was not seen in every experiment of the kind. We may summarise a few of the varieties of result obtained in a long series of experiments, by indicating once for all that the tests for the different clotting-factors were made according to the following scheme:

Test for thrombin.

Fibrinogen 5 + saline 10 + specimen 4.

Test for prothrombin.

Specimen 5 + calcium 1 + kinase 2. After 10 minutes at 37°, add fibrinogen 5 + saline 5.

Test for kinase.

Prothrombin 5 + calcium 1 + specimen 2, and then as for prothrombin.

Test for antithrombin (on solution heated to 60°).

Fibrinogen 5 + specimen 7 + saline 5 + thrombin 1. (If the specimen was oxalated, calcium was added as well.)

EXPERIMENT 57.

A sample of the same fowl-plasma as used for Expts. 52 and 53 was oxalated, and then dialysed under pressure against distilled water, till all excess of oxalate was removed. It was brought back to the original volume with 1 % NaCl, and then centrifuged clear. It showed no tendency to spontaneous coagulation, even on adding calcium. It was treated with chloroform for half-an-hour, being kept ice-cold during the treatment. The watery layer contained no preformed thrombin, but, when calcium was added, it clotted fibrinogen after half-an-hour. If kinase were added as well (test for prothrombin) the combination clotted in 30 seconds. This watery layer, however, contained no trace of demonstrable antithrombin. The tube containing it clotted simultaneously, in 4 minutes, with the control tube containing an equal addition of saline; while the tube to which the oxalated and dialysed plasma, heated in parallel, was added, began to show a weak clot only after 53 minutes. The fact that the watery layer showed a trace of activation with calcium only could be fairly attributed to its containing a trace of kinase with its prothrombin. This view was supported by the discovery of kinase in the chloroform-layer. When four drops of the extract from the chloroform-layer were added to five of the watery layer, and the mixture incubated with calcium, it clotted fibrinogen in 9 minutes.

EXPERIMENT 62.

A sample of plasma from another fowl was oxalated, and was treated with chloroform in the cold for 15 minutes. A non-oxalated sample was treated in exactly the same way, and the two watery and chloroform-layers obtained were subject to a parallel series of tests. The watery layers of both contained abundant prothrombin, clotting fibrinogen in 15 seconds after preliminary treatment with kinase and calcium. The oxalated sample contained no thrombin, but a weak thrombin was present in that treated without oxalate (clot in 22 minutes). The chloroform-layers showed a stronger thrombin (6 minutes) in the case of the non-oxalated, none in that of the oxalated sample. Both contained kinase and prothrombin, so that the chloroform-layers, in this experiment presented a rather complex problem.

No antithrombin could be detected in either layer, with either sample.

EXPERIMENT 65.

The same plasma as above was treated, with and without oxalate, with chloroform for 1¼ hours at 37°. The watery layers were tested for thrombin, with the result that the sample without oxalate caused clotting in 1 minute, while the oxalated sample had produced no clot in an hour. Neither sample contained any antithrombin, clotting occurring simultaneously in the control, and in the tube containing the two samples. The time in each case was 3 minutes, and the three clots were indistinguishable in firmness. In a fourth tube, with the heated original plasma, a weak clot began to form after 30 minutes, and was still soft after several hours. The oxalated sample contained a fairly active prothrombin, causing clotting in 2½ minutes after treatment with kinase and calcium. Yet, though it contained no antithrombin, it was not activated by calcium alone; the tube was watched for 38 minutes and then abandoned, as no sign of clotting had appeared.

EXPERIMENT 70.

This is quoted as an extreme instance of the variation in distribution of clotting-factors. The first experiment of the detailed series (No. 53) showed, in the case of an oxalated plasma, pure prothrombin in the watery, pure kinase in the chloroform-layer. In this instance, that of a plasma kept frozen for three days after bleeding, and then treated with chloroform for 1 hour at 37°, we find the exactly converse distribution. The finding of prothrombin and kinase, but no thrombin, although the plasma was not oxalated, is especially anomalous. We can only suggest that the precipitation of the prothrombin with the curdy emulsion, which occurred in this instance owing to some unknown condition, protected it from activation by the liberated kinase, which, owing to some equally unknown condition, appeared in the watery-layer when separation was effected. Whatever be the cause, the watery-layer by itself had no effect on fibrinogen, and was equally inert after treatment with kinase and calcium; when, however, it was digested with prothrombin and calcium, the mixture produced clotting in 1½ minutes, showing an effective kinase. The chloroform-layer was inactive by itself, and did not activate prothrombin, but caused clotting in 3 minutes after it had been treated with kinase and calcium, showing that it contained prothrombin.

Hitherto we have quoted instances in which at least one of the layers, water or chloroform, obtained by the treatment described, contained one of the recognisable clotting factors, practically uncontaminated by others. We met with other cases in which the conditions were less simple. We have records of experiments, for example, in which both layers had some immediate thrombic action, but showed evidence of the presence, in addition, of both prothrombin and kinase, in that their thrombic action was increased by incubation with excess either of kinase or prothrombin, in presence of calcium. A puzzling feature in the behaviour of such samples was that simple incubation, with calcium only, did not greatly increase their content of thrombin; indeed, if incubation were protracted, their activity rather declined. This seems difficult to reconcile with the presence of prothrombin and kinase in them. We believe that the clue to this anomaly is furnished by the fact, which early came to our notice, that the preparations either of prothrombin or of thrombin, but more especially of the former, obtained by the chloroform method, are remarkably unstable; the kinase, by contrast, being remarkably stable under the same conditions. While the prothrombin or thrombin may show a very high potential or developed activity when first obtained, it shows a very rapid decline even at laboratory temperature, which decline is greatly accelerated by incubation at 37°. One experiment, which we have already quoted, showed, as the final stage of this degeneration, a preparation after two days' incubation, in which only kinase could be recognised. We must suppose then, that the solution in which thrombin, prothrombin and kinase can be detected, contains also some factor by which prothrombin and thrombin are continually being destroyed, the former with great rapidity; and that this action is greatly accelerated by incubation. The thrombic action detectable at any period can probably be increased, therefore, either by accelerating the formation of thrombin, by adding extra kinase before the incubation, or by supplying excess of prothrombin, so that the formation of thrombin, by the kinase already present, does not fail through lack of the rapidly vanishing prothrombin. What, then, is the cause of this peculiar instability of prothrombin and thrombin thus prepared? A hint was afforded by the observation that many of the clots obtained by the use of these preparations, while hardening rapidly on formation, subsequently softened again, and might be completely dissolved in the course of a few hours' incubation. The solution was not a reversal of the clotting process; the mixture now yielded no further clot on the addition of thrombin, and gave no coagulum on heating to 60°. Clearly the initially formed fibrin had been digested. This might, perhaps, have been anticipated from Jobling's demonstration of the liberation of a tryptic ferment in serum shaken with chloroform—an action which we have repeatedly confirmed. And this tryptic action, ensuing on the destruction of the antitrypsin by the action of chloroform, explained not only the instability of the thrombin or prothrombin obtained by this method; it furnished also a possible clue to the appearance of kinase, the resulting activation of the prothrombin, and the whole series of phenomena under discussion. We proceeded to put this possibility to the test, by treating fowl's plasma with trypsin.

Effect of Trypsin on Fowl-plasma.

The trypsin used was the dried preparation of Fairchild, and this was made up as a 1 % solution in water as required. The sample of fowl-plasma used was that employed in Experiment No. 62. This plasma, at the time of the experiment with trypsin, had been kept in the cold-room for a week.

A few preliminary experiments showed that the trypsin itself had no coagulant effect on fibrinogen, and no activating effect on prothrombin in the presence of calcium ions. It contained, therefore, neither thrombin nor kinase.

Like all specimens of natural plasma or serum, the fowl-plasma possessed a marked antitryptic action, and it was necessary, in the first instance, to discover the amount of trypsin required to overcome this effect. It was found that five drops of the 1 % trypsin solution, added to 10 cc. of the plasma, had no apparent effect on the clotting properties during incubation for 1 hour. At the end of that period, a sample of the mixture was as free from activating effect, when added to prothrombin with calcium, as the original plasma was. When the proportion of trypsin was increased ten times, however, the effect was very soon apparent.

EXPERIMENT 80 (*b*).

To 1 cc. of fowl-plasma were added 5 drops of 1 % trypsin, and the mixture placed in the water-bath at 37°. Samples were removed at intervals, and tested according to the usual scheme for kinase, as follows:

Prothrombin 5 + sample 2 + calcium 1, incubated 10 minutes; then added:

Fibrinogen 5 + saline 5.

The following results were obtained:

With original fowl-plasma.—No clot after several hours.

With sample after 3 minutes' tryptic digestion.—Hard clot in 5 minutes.

With sample after 6 minutes' tryptic digestion.—Hard clot in 2 minutes.

With sample after 15 minutes' tryptic digestion.—Hard clot in 1½ minutes.

There is clear evidence, therefore, of the production of kinase by the action of trypsin on the plasma. The question at once arises, however, why the formation of the kinase does not result in the clotting of the plasma itself, since the latter contains fibrinogen, prothrombin and calcium. As a matter of fact, the plasma does clot eventually. In the experiment above recorded, the residue of the plasma-trypsin mixture, left in the water-bath, set to a soft clot 5 minutes after removal of the last sample, and therefore 20 minutes after the commencement of the incubation. This clot did not harden, but, on the contrary, soon showed signs of dissolution, and had nearly disappeared at the end of 3 hours. Evidently we were dealing with a complex action of the trypsin, which throws considerable light on the anomalies encountered in dealing with the action of chloroform. The trypsin not only sets free kinase, but simultaneously digests prothrombin, thrombin, fibrinogen, and, later, the fibrin, if any should be formed.

Since several authors have shown that the temperature-coefficient of the action of thrombin above 17° is very small, while that of trypsin is much larger, it occurred to us that the excess of thrombin formed over that destroyed, at any period of the digestion, might be demonstrated by removing samples from the warm bath, and allowing them to cool to the room temperature. This expectation was verified as the following record shows.

EXPERIMENT 80 (*a*).

5 cc. of fowl-plasma + 25 drops of 1 % trypsin, were placed in the bath at 37°, and samples of 0·5 cc. removed, at intervals, to separate tubes, and

allowed to cool. The times indicated are the intervals after removal from the bath at which clotting occurred.

Sample removed immediately.—Did not clot in 3 hours.

Sample removed after 3 minutes' incubation.—Did not clot in 3 hours.

Sample removed after 6 minutes' incubation.—Clotted hard in 22 minutes.

Sample removed after 15 minutes' incubation.—Clotted hard in 5 minutes. The remainder clotted softly after 20 minutes, without removal from the warm bath, as described above.

In another experiment, using only one-half the proportion of trypsin giving the above results, we detected no production of kinase or other change in the plasma after 30 minutes' incubation. The proportion of trypsin was then increased to that used above (5 drops of 1 % solution to each cc. of plasma) and the formation of kinase, with clotting of the plasma on cooling to room-temperature, occurred as before.

In another experiment the plasma was oxalated, and 1 % trypsin added in the proportion of 5 drops to 2 cc. of plasma. Again nothing occurred after 30 minutes' incubation, and again, after adding a further equal proportion of trypsin, so as to bring the total up to that which proved active with unoxalated plasma, kinase was formed after a further 10 minutes' incubation, as the following test shows (Exp. 84):

Prothrombin 5 + sample 2 + calcium 1, incubated 10 minutes; then added:
  Fibrinogen 5 + saline 5.—Clotted hard in 4 minutes.

Naturally, in the absence of calcium ions, no clotting of the plasma occurred on cooling. Nor could any thrombin be detected by adding four drops to the usual fibrinogen-dilution.

It is quite evident that the results of treating fowl-plasma with trypsin are very similar to those obtained with chloroform, as described in the foregoing section. In both instances we have the liberation of kinase, which, if calcium is present, acts on the prothrombin of the plasma to form thrombin. With the knowledge that one effect of chloroform on plasma is to liberate trypsin, it seems justifiable to regard this as the primary action, to which the appearance of kinase and thrombin are secondary. It then becomes easy to understand why the effect of chloroform, on one batch of plasma, should vary with the time which elapses between the preparation of the plasma and the performance of the experiment. The fact, for example, that a plasma which, when fresh, readily gives a powerful thrombin on treatment with chloroform, may, after cold storage for a week or two, give only prothrombin, readily finds its explanation. In both instances the chloroform treatment removes the antithrombic and antitryptic actions of the plasma. In the fresh plasma, active trypsin is thereby unmasked, with a resultant production of kinase and, unless calcium has been removed, of thrombin; in the older plasma, we must suppose that the trypsin has undergone decay, so that the only perceptible action of the chloroform is to remove antithrombin, and precipitate fibrinogen, leaving a solution in which prothrombin is the only clotting-factor which can be detected.

THE SUPPOSED ANTAGONISM BETWEEN KINASE AND ANTITHROMBIN.

Howell's [1911] conception of the function of kinase (thromboplastin) starts from the assumption that the substance, or substances, in plasma, which hinder the clotting action of formed thrombin, also hinder the activation of prothrombin by calcium. As we have already indicated, we can find no ground for this assumption, though it is peculiarly difficult to disprove. For the only test of the activation of prothrombin, namely, the appearance of thrombic activity, cannot be applied in the presence of substances by which that activity is inhibited. Howell, however, who considers the function of kinase (thromboplastin) to be the removal of antithrombin, permitting the activation of prothrombin by calcium, supports his conception by evidence designed to show that kinase neutralises the inhibitory action of normal or peptone-plasma, and of hirudin, on the action of formed thrombin.

It will be clear that, if evidence of this effect is to be unequivocal, it must be obtained under conditions which allow the balance between thrombin and antithrombin to be upset in only one way, namely, by the neutralisation of the antithrombin. If there is any possibility of an increase in the amount of thrombin present, the experiment appears to us of no value for the purpose. This criticism would exclude a considerable part of Howell's evidence on this point, in that the experiments were made with oxalated plasma, with the addition of calcium salts. According to Howell, "the acceleration in the time of clotting caused by the thromboplastin cannot be explained by assuming that it served to activate the prothrombin in the oxalated plasma, since the control experiment with calcium chloride showed that this substance alone was sufficient for the purpose." The control experiment in question showed, indeed, that, when calcium chloride was added to the oxalated plasma, sufficient thrombin was formed to clot the plasma in nine minutes. But it surely furnished no proof that the whole of the prothrombin was activated in that time, and that the addition of tissue extract would cause no increase in the amount of thrombin formed. There is at least a possibility here of the balance between thrombin and antithrombin being upset by addition of thrombin, rather than removal of antithrombin.

The same criticism applies with even greater force to the experiments illustrating the activation of "a preparation containing both prothrombin and antithrombin" by tissue extract. To assume that the action, in this case, is a removal of antithrombin, would be to beg the whole question at issue.

If these experiments are excluded, as giving results which are explicable, with at least equal ease, on the Morawitz theory, there is left, of Howell's evidence, only the series in which fibrinogen solution, or oxalated and dialysed plasma were used, without addition of calcium. In this series the "antithrombin" used was not heated plasma, but hirudin. And the nett result of the experiments is to show that, while a proportion of hirudin, which neutralises the action of thrombin indefinitely, generally does so still, if tissue extract be added as well, smaller proportions, which greatly delay the action of thrombin, generally delay it to a smaller extent in the presence of tissue extract. The degree, in which the tissue extract weakens the hirudin action, may be gathered from the clotting times of parallel experiments.

Hirudin	With tissue extract	Without
1 drop	Less than an hour	2 hours 15 minutes
2 drops	3 hours	6½ hours (weak)
3 „	No clot	No clot
2 „	30 minutes	2–3 hours (imperfect)
4 „	2 hours 15 minutes	No clot in 24 hours

There are other experiments in which the difference produced by "thromboplastin" is much less than this. But, in the best case, what does it amount to? The effect of hirudin, sufficient to prolong clotting beyond 24 hours—say, for practical purposes, indefinitely—is so weakened, that clotting occurs after little more than two hours. But the effect, which the action thus demonstrated is supposed to explain, is something of a different order altogether. A fowl-plasma, for example, which remains stable for weeks or months, clots in a few minutes if some kinase be added. When, further, the notorious instability of hirudin is remembered, together with the presence of proteolytic ferments in tissue extracts, it becomes clear that there are several possibilities needing exclusion, before evidence like the above can be accepted as showing any genuine antagonism between thrombokinase and the antithrombin of plasma.

In our own experiments, made to test the existence of such an antagonism, we attached great importance to the exclusion of prothrombin from all the reagents used. We are testing the resultant action of a mixture of thrombin and antithrombin. If any prothrombin is present, and calcium not excluded, the addition of kinase will increase the balance in favour of thrombin. We may describe in detail an experiment in which these precautions were observed.

Our fibrinogen was a sample which had been rigidly tested in this direction, and found to be indefinitely stable with calcium and kinase. The antithrombin, obtained by heating fowl-plasma to 60° for 20 minutes, and filtering from coagulated fibrinogen, was subjected to a thorough test. Four tubes were put up, each containing 5 drops of fibrinogen solution, 2 drops of kinase, and 1 drop of calcium chloride (1 %). To these we added 1, 2, 3 and 4 drops respectively of the heated fowl-plasma. There was no sign of clotting in any tube after 24 hours. Therefore, so far as traces of prothrombin can be detected in the presence of antithrombin, we may conclude that the sample was prothrombin free.

The thrombin was obtained by shaking fresh fowl-plasma with chloroform and digesting for 1 hour at 37°. It was an extremely active specimen, and needed diluting many times to render its clotting-time susceptible of accurate measurement. After diluting 10 times, 1 drop clotted the usual fibrinogen dilution in 20 minutes. Two tubes were put up with this amount of thrombin and fibrinogen; to each was added one drop of calcium chloride, and to one of them 2 drops of kinase. Both clotted simultaneously. We may conclude, therefore, that the thrombin contained no remnant of prothrombin. That it contained traces of kinase is probable; but that would not affect the validity of the experiment.

After a few preliminary tests, to get a suitable mixture of thrombin and antithrombin, we put the following mixture into a tube and placed in the bath at 37°:

Fibrinogen 6 + antithrombin 15 + 4 times diluted thrombin 1 + calcium 1. After 10 minutes' incubation we divided the solution into two equal portions, added 2 drops of kinase to one, and returned both to the water-bath. It is peculiarly difficult to time accurately the beginning of the clotting-process in the presence of antithrombin, which not only delays the onset of the process, but also makes the hardening of the clot much slower. No difference, however, could be detected between the two tubes in this experiment. In both, the first signs of increasing viscosity occurred about 20 minutes from the first mixing, and the clots in both tubes were equally firm at 25 minutes.

A similar experiment was made, increasing the antithrombin to 20 drops, with 1 drop of diluted thrombin as before. After 5 minutes' incubation the mixture was divided again, 2 drops of kinase being added to one-half, and 2 drops of 30 % glycerol to the other. In each tube the first thickening began after about 40 minutes, and the clot became firm in about 50 minutes. Several similar experiments were performed, using fibrin-extract as thrombin, and heated rabbit or guinea-pig serum as antithrombin. In no case could we detect any definite acceleration of the clotting process by kinase. Yet the proportions of kinase were such as, when added to indefinitely stable fowl-plasma, produced clotting in a few minutes.

We are compelled, therefore, on this point also, to range ourselves with the earlier observers (Morawitz, Fuld and Spiro), and in opposition to Howell, in our inability to detect any real antagonism between kinase (thromboplastin) and antithrombin.

Howell's Prothrombin.

One of the greatest difficulties of any discussion on blood-clotting is the use of the current nomenclatures without prejudice to the point in dispute. In accordance with the original employment of the term by Schmidt, we have used "prothrombin" to denote the inactive precursor of thrombin, which is activated by kinase in the presence of calcium. Howell regards a preparation showing these properties as a complex of the thrombin-precursor with antithrombin. It is necessary to remember that, having supported this view of the constitution of prothrombin, by evidence which we have already discussed, Howell thereafter applies the name "prothrombin" to preparations which he regards as free from antithrombin, since they need only calcium for their activation. Such a preparation he obtained from cat-plasma by precipitating with acetone, washing the precipitate with ether, and drying in a current of warm air. Though we have followed Howell's directions as closely as possible, we have hitherto failed to obtain from rabbit-plasma a preparation yielding thrombin on treatment with calcium alone, though our preparation was readily activated when treated with kinase in addition. We found no evidence of antithrombic action after the preparation had been heated to 60° for 4 minutes. We hope to be able to obtain a preparation corresponding in properties to Howell's, and propose, in that event, to examine it for the presence of kinase by use of the fibrinogen and prothrombin preparations which we have employed in our other experiments. It is clear that the value of the evidence, as to the properties of such a preparation, is dependent on the proved freedom from prothrombin of the fibrinogen used. If the test-fibrinogen were contaminated with prothrombin (in the original sense), it is evident that a solution containing only kinase would exhibit the properties of Howell's "prothrombin." And on this particular point, of the freedom of his fibrinogen from prothrombin, Howell's published evidence seems to us to be incomplete. His fibrinogen is described as being prepared by precipitation and reprecipitation from oxalated cat-plasma, with an equal volume of saturated sodium chloride. Rettger's precaution of clarifying the solution, by forming a filmy precipitate of barium phosphate—which possibly would produce the same effect as the barium sulphate used by us—was found unnecessary, since, without it, solutions were obtained which did not clot spontaneously on adding calcium. There is no evidence that they were tested with kinase in addition. This omission seems to us to weaken all the evidence obtained with such preparations; for to assume that prothrombin, if present, would be activated by calcium only, is to assume the point which the solution is used to demonstrate at a later stage of the investigation.

SUMMARY AND DISCUSSION.

The following are the main experimental results of this preliminary investigation.

1. Indications have been obtained for the preparation of a set of test-solutions of the different factors concerned in the coagulation of blood-plasma. In particular it has been found possible to prepare

(*a*) Fibrinogen, free from perceptible traces of either prothrombin or kinase, from oxalated mammalian plasma, by adsorbing prothrombin with barium sulphate, and then precipitating the fibrinogen by half-saturation with sodium chloride.

(*b*) Prothrombin, free from perceptible traces of thrombin or anti-thrombin, by a modification of Mellanby's method.

2. Fresh fowl's plasma, when shaken with chloroform, readily yields a powerful thrombin. If the plasma be previously freed from calcium, prothrombin and kinase are formed by the same treatment, and can often be obtained apart by centrifuging the chloroform from the watery residue. If the treatment with chloroform be continued for some days at 37°, kinase is the only clotting-factor remaining.

3. A similar formation of kinase and (in the presence of calcium) thrombin occurs, when fowl's plasma is treated with an appropriate proportion of trypsin. The effect of chloroform is probably due, therefore, to the liberation of the normal tryptic ferment of the plasma, by the destruction of antitrypsin.

4. We have failed to confirm the antagonistic action of kinase on antithrombin, described by Howell.

So far as these results have an incidental bearing on theories of blood-coagulation, they seem to us to be definitely in favour of the necessity of three factors for the formation of thrombin—prothrombin, kinase and calcium. They are not compatible with the more recent conception of Howell, according to which the function of the kinase is to inactivate the antithrombin, which prevents the activation of prothrombin by calcium.

We may turn from the results of experiment, and conclusions directly deducible therefrom, to some considerations of a more speculative nature.

For the more direct purposes of our investigation, the point of most significance, and of most novel interest, is the demonstration of the appearance of kinase, as the result of slight tryptic digestion, in a body-fluid which previously contained none. It seems possible that this phenomenon will prove to be of considerable physiological interest. Hints of such fermentative production of kinase can be found in earlier observations—for example, in Howell's statement that a tissue yields a more powerfully kinasic extract, if it be allowed to undergo some degree of autolysis at room temperature before extraction. It may be that some degree of protein cleavage is the necessary preliminary to the liberation of kinase from any tissue; and that the ease with which it appears on cell-injury, and the relatively drastic treatment needed for its production in a stable avian plasma, represent a differing delicacy in the poise of the balance between proteolytic ferment and those antagonistic influences which, in the case of the plasma, we summarily describe as "antitrypsin."

Much further experiment is needed to define the relation between this liberation of thrombokinase, and the toxicity acquired by serum and other native proteins, when subjected to a similar partial hydrolysis, and speculation on the basis of existing results would be premature. We have, as yet, no evidence as to which constituent of the plasma is involved in the hydrolytic production of kinase, though evidence, on the one hand, as to association of kinasic action with a lecithin-like substance (according to Howell with the kephalin-fraction of tissue-lipoid), and, on the other hand, as to the presence of such a constituent in the euglobulins, must be regarded as suggestive. To this suggestion, and to the relation between kinase-formation and the toxicity of variously treated sera and proteins, including the specific toxicity of foreign protein to the anaphylactic animal, we hope to return in future communications.

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XXX. CREATINURIA IN CHILDREN.

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It has been known for some years that creatine is constantly found in the urine of young children although none appears in normal adult urine, or at any rate in that of male adults. As yet nothing appears to be known concerning the cause of this difference.

Rose [1911], who examined specimens from a number of children and young people, found that creatine was usually present until the age of puberty. His observations were not made on 24-hour specimens of urine, and in many cases the diet contained meat. Folin and Denis [1912, 1] also made observations on a number of normal healthy children, and found that the creatine formed a fairly large fraction of the "total creatinine" excreted, even in the case of vegetarians. It was also shown that the ingestion of meat was followed by a rapid rise in the creatine output. They made a few observations on morning, afternoon and night samples, but these did not lead them to any conclusions concerning the relative amounts of creatine excreted during different parts of the day. Krause [1913] has also made observations on the creatine excretion of children, and concludes that creatine occurs in the urine of normal boys up to about six years of age, and for a longer time in the case of girls. To determine the fate of ingested creatine he gave two boys 0·3 g. of creatine in the morning. One, aged eight, who was previously excreting no creatine, excreted 43 % of that given, and the other, aged five, excreted at least 22 % of that given (one sample of urine being lost). Approximately the same amount was given to two girls the last thing at night; one, aged eleven, previously excreting no creatine, excreted during the next 24 hours 31 % of that given, and the other, aged six, excreted 56 %. In no case was the ingestion of the creatine followed by any apparent increase in the excretion of creatinine.

According to some observers, e.g. Plimmer, Dick and Lieb [1909], adults can take as much as 2 g. of creatine per day without any of it appearing in the urine, as such or as creatinine, though the more recent results of Myers and Fine [1915] show some excretion by one subject when only 1 g. was taken, and there was apparently a slight conversion of creatine to creatinine by both subjects when larger amounts of creatine were taken.

The starting point of our own experiments was a case of amyotonia congenita on which we were making a fairly detailed metabolism study. The urine was found to contain a very large amount of creatine relative to the creatinine, and in order to study the relationship more closely the separate samples of urine passed during the 24 hours were examined. For comparison a similar study was then made on a normal boy.

The determinations of both creatinine and creatine were made essentially by the new Folin [1914, 1] method. One or two points with respect to this method noticed during our investigation may be mentioned here. When the urine is so dilute that more than 5 cc. are required for 1 mgm. of creatinine, Folin recommends diluting the standard also before adding the alkali. If, however, the urine is so dilute as to require 15 cc., a case we sometimes encountered (after making to a suitable volume), this method is not very convenient if a number of observations have to be made on one day; for under these conditions the colour is at first about 15 % less than if the same amount had been present in 1 cc., but increases on standing, so that after 3 hours the readings would be much more nearly equal. We therefore diluted the standard creatinine solution to 5, 8, 12 and 15 cc., and compared the colorimeter readings at between 3 and 9 minutes after making to volume with that of the standard where no water had been added, this being set at 1·00 cm. The readings were 1·015, 1·02, 1·06 and 1·19 respectively. With these figures we were then able to correct the readings when using any volume of urine up to 15 cc., without requiring to make a new standard for each determination.

McCrudden and Sargent [1916] have recently thrown doubt on the accuracy of the new Folin method of estimating creatine in urine, since they find that this method gives higher results than the hydrochloric acid conversion, and gives some "apparent creatine" in almost every urine, though all the figures quoted seem to be from nearly normal patients. Our experience does not agree with that of McCrudden and Sargent. With a sample of normal adult urine a reading of 0·99 cm. (mean of four settings of colorimeter) was found for creatinine; exactly the same reading was found after treatment for conversion of creatine by the new Folin method (except that broken glass was substituted for pebbles), whereas the hydrochloric acid method gave a reading of 1·01, i.e. apparently a slight loss, but within the limits of error. We have nearly always used a few small pieces of porous pot to facilitate steady boiling. A disadvantage of this is that after adding the alkali a rather gelatinous precipitate is formed. But if after making to volume one allows the mixture to stand for 10 minutes, this settles sufficiently to enable enough clear solution to be pipetted from the top. If one prefers to centrifuge, the solution used for the estimation of the preformed creatinine should also be centrifuged, as the colorimeter reading is about 3 % higher after centrifuging, which, though almost negligible for the preformed creatinine, may considerably influence the creatine figure.

In the creatine estimation we have always made the volume to 20·5–21·5 cc. before adding the alkali in order to avoid any errors caused by differences in dilution, since the correction for dilution used for creatinine is not suitable for creatine if one desires to wait for the precipitate to settle. For most of the determinations in urine for which McCrudden and Sargent give details the volume of urine and picric acid solution was 23 cc. in the creatinine determination, 20 cc. in that of creatine by the new Folin method, and at least 25 cc. where hydrochloric acid was used. These differences in dilution are in the order which, according to the results given above, would give an "apparent creatine" value, but we should expect any error due to this to be not greater than 2 % of the creatinine value, whereas in many cases their "apparent creatine" is much higher than this.

In our further determinations to test the method of creatine estimation, a sample of normal adult urine, for which when using 1 cc. the creatinine reading was 0·58 when centrifuged (0·555 not centrifuged), read also 0·58 when centrifuged after treating by the new Folin method for creatine, using porous pot. A sample from another normal adult read 1·11 for creatinine (1·085 when not centrifuged) and for creatine 1·105 when boiled for 2 hours, and 1·11 when boiled for 5 hours, and both centrifuged. It is seen that our results with three different samples of adult urine, all quite normal so far as known, do not agree with McCrudden and Sargent's, which, at any rate for the most part, were obtained on nearly normal urine; and if it is granted that normal male adult urine contains no creatine, our results are quite satisfactory.

In connection with the creatine estimation we noticed that after the urine had been boiled with picric acid, the development of the colour on adding the alkali appeared to proceed much more rapidly than when the mixture had not been boiled. This was not due simply to a difference in temperature, since we usually allowed the solution used for creatine estimation to stand overnight after boiling and making to 21 cc., the creatinine determination being made at the same time.

The volumes of urine taken for the estimation of the creatinine and creatine in any one sample were always such that the readings were very nearly the same, so as to avoid any error due to the reading not being exactly proportional to the amount present. Throughout the paper and on the curves the creatine values are all given as their equivalent of creatinine.

For convenience the results with the normal boy will be dealt with before those from the amyotonia case. The child investigated was three years old and the diet was free from meat and meat extract. Meals were taken each day at about 8 a.m., 1 p.m. and 6 p.m. In the first period the collection extended over four days (Jan. 4–8) and in the second over eight days (Feb. 27–Mar. 5). To give the results for all the samples passed during these periods would occupy too much space, and on account of the bladder not being emptied at the same time each day it is almost impossible to give an average figure for the different parts of the day. Curves showing the amounts of creatinine, creatine and total nitrogen excreted per hour during the 4-day period, as well as the percentage of "total creatinine" excreted as creatine, are given in Fig. 1[1]. The most significant fact shown by these curves is the very small excretion of creatine during the night, whether this is measured in absolute amount or as percentage of the "total creatinine." It will also be observed that, measured in either way, the amount is larger on the whole for the morning than the afternoon.

The excretion of creatinine per hour during the night is also seen to be lower than the average during the day-time, but only slightly so. The curve for total nitrogen is similar in character to those for creatine and creatinine, showing less variations between day and night than the creatine but greater than the creatinine. The curve representing volume per hour (not inserted in Fig. 1) runs fairly parallel to that for creatine, though exceptions have been observed both in this and in subsequent periods.

The child slept soundly from a short time after the beginning of the night period to within a short time of its close. It may be pointed out that the amount of creatine excreted during the whole of the night period averages only 7 mgm., an amount approximately equal to that during the last 1½ hours before bed or the first hour during the morning. Consequently a large part of that found during the night period may have been excreted before going to sleep or after waking, and not during the actual sleep period. On the first and third days he also slept for about 2 hours during the afternoon, but this does not seem to have affected the results appreciably.

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[1] The daily creatinine values for this period average 254 mgm. In two succeeding periods they were found to be 231 and 239 mgm. The improbability of this variation led to an examination of the standard creatinine solution being made, and it was found to have undergone some decomposition. The values for creatine and creatinine in this period are therefore probably 7 % too high.

The results obtained during the 8-day period were quite in agreement with those given in Fig. 1, except that on only one day was the creatine output during the last period before going to bed greater than during the preceding period. At 8 p.m. on the third day 120 cc. water were given, and at the same time on successive days, 130 cc. milk, 16 mgm. creatine (= 13·8 mgm. creatinine) in 40 cc. water, and 31·4 mgm. creatine (= 27·1 mgm. creatinine) in 50 cc. water, but apparently none of these had any influence on either the creatine, creatinine, total nitrogen, or volume excreted, these all being normal as judged by the two preceding and succeeding nights and days.

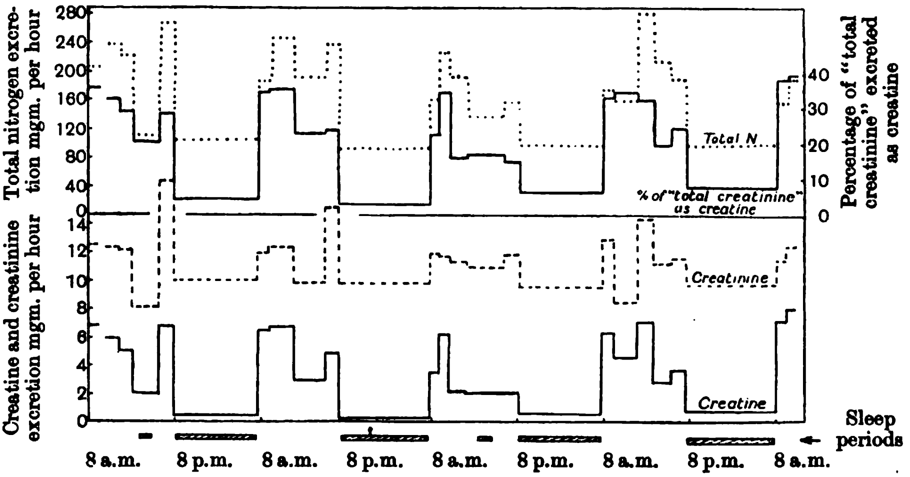

Fig. 1.

It was then recognised that although the child may be able to deal with an appreciable quantity of creatine when given at the beginning of the night period without any of it being excreted, yet this may not be so when it is given at the beginning of the day period, since during this time a relatively large amount of the total creatinine is excreted as creatine, indicating either a greater rate of production or smaller rate of utilisation, or both, compared with the night period. The following experiment shows fairly conclusively that the difference in the power of utilisation is an important factor. Observations were carried out for six days, from 7 a.m. on April 3rd to 7 a.m. on April 9th, and the urine for each day was collected in three periods, 7 a.m to 1 p.m., 1 p.m. to 7 p.m., and 7 p.m. to 7 a.m., the bladder being emptied at a time very near the end of each period. On April 7th it was noticed that the child had developed a slight cold, but there was no fever. At 7 o'clock on the *evening* of the third day 102 mgm. of creatine (= 88 mgm. creatinine) were given by mouth in 30 cc. water, and at 7 on the *morning* of the sixth day this dose was repeated. The curves in Fig. 2 show the rate of creatinine and creatine excretion, in mgm. per hour, during the six days, as well as the percentage of the "total creatinine" present as creatine. From the curves it is easily seen that the creatine when given in the evening has no appreciable influence on the excretion of either creatine or creatinine, but when given in the morning there is considerable increase in the creatine excretion during the next 12 hours, but none in the creatinine excretion. The following Tables (I and II) emphasise the points just mentioned, the

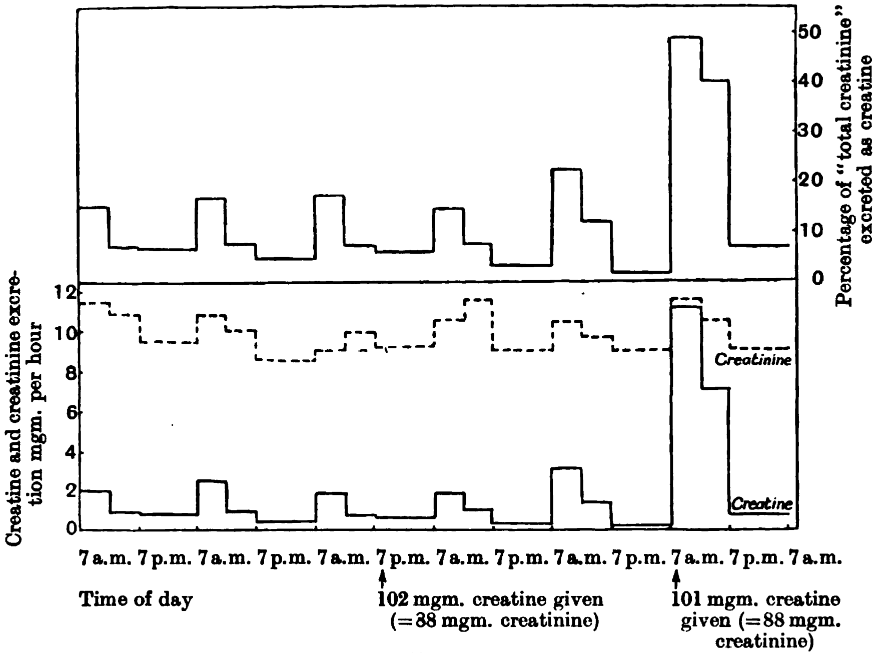

Fig. 2.

second and third lines giving the rate of excretion up to 24 hours after taking the creatine, and the first line the average values for the rest of the period.

TABLE I.

Creatine excretion. Mgm. per hour.

	7 a.m. to 1 p.m.	1 p.m. to 7 p.m.	7 p.m. to 7 a.m.
Average for normal days ...	2·3	0·94	0·43
Creatine (=88 mgm. creatinine) given at 7 p.m.	1·8	0·95	0·59
Creatine (=88 mgm. creatinine) given at 7 a.m.	11·1	7·0	0·68

TABLE II.

Creatinine excretion. Mgm. per hour.

	7 a.m. to 1 p.m.	1 p.m. to 7 p.m.	7 p.m. to 7 a.m
Average for normal days ...	10·4	10·1	9·0
Creatine (=88 mgm. creatinine) given at 7 p.m.	10·5	11·5	9·15
Creatine (=88 mgm. creatinine) given at 7 a.m.	11·5	10·5	9·05

The figures for each part of the 24 hours after taking creatine are all within the limits of variation for normal days except those for the morning and the afternoon period after its administration at 7 a.m.; during these the excess above the normal excretion was 50 mgm. from 7 a.m. to 1 p.m. and 37 mgm. from 1 p.m. to 7 p.m., giving a total of 87 mgm., that is, equal to the amount given.

It was noticed that the urine for the morning period beginning 24 hours after the last administration of creatine contained an appreciable amount of acetone and a trace of aceto-acetic acid, and the creatine content was higher than normal. Acetone had never been found in any of the previous samples of urine from this child. In order to be quite certain that this high creatine excretion was an adventitious circumstance associated with the slight acidosis, the urine was again collected for 4 days beginning 7.30 a.m. April 18th. At 7.30 on the morning of the third day, 99 mgm. of creatine (= 86 mgm. creatinine) dissolved in 30 cc. water were given. As in the preceding period the creatine excretion was greatly increased during the ensuing morning and afternoon as shown by the results in Table III.

TABLE III.

Excretion of creatine. Mgm. per hour.

	7.30 a.m. to 1.30 p.m.	1.30 p.m. to 7.30 p.m.	7.30 p.m. to 7.30 a.m.
Average of 1st, 2nd and 4th days	6·5	2·7	0·90
3rd day	13·9	4·3	0·34

On this occasion there was no acetone in the urine on the morning of the fourth day, and the creatine was quite normal in amount, showing that the acetone and high creatine on the preceding occasion were not due to the creatine taken on the preceding morning.

The amount of creatine excreted during the third day in excess of the normal for this period was 45 mgm. from 7.30 a.m. to 1.30 p.m. and 10 mgm. from 1.30 p.m. to 7.30 p.m., giving a total of 55 mgm., each reckoned as creatinine. In this period we see that less than two-thirds of the creatine given in the morning was recovered in the urine, whereas in the preceding period apparently the whole amount was recovered.

In adults the excretion of creatine is usually associated with acidosis, in consequence of which a larger percentage of the total nitrogen is excreted as ammonia than by normal adults. In order to see how far similar conditions held in the child under investigation, the ammonia and the total nitrogen were estimated in each sample of urine during the last period, using the van Slyke and Cullen [1914] method for ammonia. Apparently the excretion of these two substances was not appreciably affected by the creatine administration.

The average results for the period, excluding the 24 hours after taking creatine, are given in Table IV.

TABLE IV.

	7.30 a.m. to 1.30 p.m.	1.30 p.m. to 7.30 p.m.	7.30 p.m. to 7.30 a.m.
Creatinine. Mgm. per hour	11·1	10·4	8·55
Total nitrogen. Mgm. per hour	184	143	86
N as ammonia. Mgm. per hour	5·43	3·26	5·27
Percentage of total N as NH_3	2·95	2·28	6·12
Percentage of "total creatinine" as creatine	37	20·6	9·5
Creatine. Mgm. per hour	6·5	2·7	0·90

It is seen that the percentage of the total nitrogen excreted as ammonia is highest at night when the creatine is lowest, and is slightly less during the morning than during the afternoon period, although the creatine is much greater.

In accordance with the ammonia results, the night urine was acid to litmus on each occasion, whereas that for the morning and afternoon was alkaline. The conditions then are quite different from those found to hold when creatine is excreted by adults. Moreover as pointed out by Folin and Denis [1912, 1], if the creatine excretion by children is due to lack of carbohydrate, one would expect it to be particularly high in the night urine, whereas our observations show that the reverse holds good.

Milk Factor. During and for one day preceding each period, the diet, as mentioned previously, was free from meat and meat extracts, and consisted chiefly of bread and butter, milk, porridge, milk pudding, vegetables, fruit, and eggs.

Folin [1914, 2], however, has found that milk contains an appreciable quantity of creatine. One sample examined by us, using the method advocated by Folin, contained 2 mgm. of creatinine and 9 mgm. of creatine (reckoned as creatinine) and another sample 2 mgm. creatinine and 8 mgm. creatine per 100 cc. Including the milk pudding and porridge, the child under examination took about 300 cc. of milk each day from the same source as the second sample, and containing therefore about 24 mgm. of creatine. Our experiments on the ingestion of creatine indicate that the fate of any creatine contained in the food will depend largely on the capability of the body for utilising or destroying it at the time that the creatine is being absorbed. This capability is best indicated by the smallness of the amount of endogenous creatine being produced at the same time. It is probable therefore that on a milk diet a portion of the creatine excreted during the day is of exogenous origin, but at night practically the whole of the exogenous creatine may be utilised.

We should like to emphasise the creatine content of milk, since it has usually been allowed when a diet free from creatine was desired.

The day and night urine for another normal boy aged 2½ years was also examined, and here again it was found that the excretion of creatine, both in absolute amount and as percentage of the "total creatinine," was less during the night than during the day. But the difference between the night and day periods was much smaller than in the preceding case, probably because this second boy awoke once or twice during the night and received 180 cc. of milk just before going to bed and another 180 cc. during the night.

The case of amyotonia congenita with which we were concerned was a girl four years old, on whom a fairly detailed metabolic study was carried out, the results of which are being published elsewhere [Powis and Raper, 1916]. In this case the daily urine contained about 67 mgm. creatinine but about 118 mgm. creatine (reckoned as creatinine), so that about 64 % of the "total creatinine" was present as creatine. As in the preceding cases, during the experiment and for 43 hours before its commencement, the diet was free from meat and meat extract, but it contained about 800 cc. of milk per day. The results on two normal days are shown by the first portion of the curves in Fig. 3. On these days the rate of creatine excretion is practically the same during the morning and afternoon, but is less during the night. The percentage of "total creatinine" as creatine is less during the afternoon than the morning, and still less at night. The creatinine excretion, judged by these and the two succeeding days, shows no definite variation with the time of the day.

It is seen that the variations in the creatine excretion during the different parts of the day are similar in character to those recorded above for the normal boy, but are much less marked, possibly because this subject spent the whole of the day in bed. The child went to sleep and awoke earlier than the normal boy and in accordance with this it will be noticed that the greater rate of creatine excretion also starts at an earlier hour, but as with the normal boy, occurs in the first period after sleep.

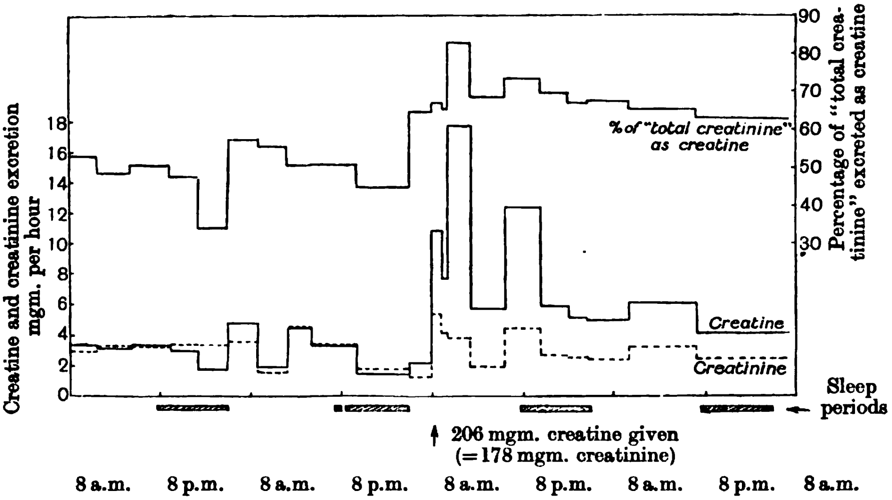

Fig. 3.

At 8 a.m. at the beginning of the third day 206 mgm. of creatine (= 178 mgm. creatinine) dissolved in 20 cc. of water were given. There was immediately a marked rise in the rate of creatine excretion and the rate continued to be higher than normal throughout the ensuing 48 hours; this is true even for the two night periods, whereas with the normal boy the administration of 102 mgm. of creatine at either 7 a.m. or 7 p.m. did not influence the creatine excretion during the night period. This finding is quite in accordance with the high creatine excretion of this subject, and shows a smaller capacity for utilising or destroying this substance. The total amount of creatine excreted during the 48 hours following its administration was 311 mgm., whereas during the preceding 48 hours 138 mgm. were excreted.

This gives an excess of 173 mgm., which is almost equal to the amount taken (178 mgm.). There is no increase in the creatinine excretion, the total amount during the preceding 48 hours being 137 mgm. and during the succeeding 48 hours 130 mgm.

Acetone was occasionally present in the urine of this subject, but there was none in any of the samples during this period of observation.

Our results on the ingestion of creatine by children are not incompatible with those of Krause, who did not compare the night and morning administration on the same case, and gave a larger amount, viz. 0·3 g.

Table V contains the data for the average amounts excreted per 24 hours on the days when no creatine was given, as well as the amounts per kilo. of body weight.

TABLE V.

Subject	Age (years)	Weight (kilogram)	Total N. (g.)	Creatinine (mgm.)	Creatine (mgm.)	% of total creatinine as creatine	Mgm. creatinine per kg.	Mgm. creatine per kg.
Normal boy F.P.R.								
Apr. 3rd to 9th	3	14	—	231	25	9·6	16·5	1·8
Apr. 18th to 22nd	3	14	3·10	239	59	20	17·1	4·2
Normal boy G.B.L.	2½	14	4·34	192	81	30	14	5·8
Amyotonia, Girl (average of three periods—in all 13 days)	4	9·7	3·73	67	118	64	6·9	12·1

The two other periods with F.P.R. gave 18·3 and 17·6 % of the "total creatinine" as creatine, but owing to some decomposition of the standard the exact amount of creatinine and creatine is not known. Assuming the creatinine to be 235 mgm. per day, the creatine in these periods was 57 and 50 mgm. per day. The figures for the normal children are in fair agreement with those found by Folin and Denis [1912, 1] and by Krause [1913] as far as comparison is possible.

The above results show the great difference between the amyotonia congenita case and normal children. Morse [1916] has recently published a statement that he has determined the creatinine and creatine in the urine of children with various diseases involving atrophy, and in all of them the creatine content was high.

It will also be noticed that the creatine excretion of the normal boy F.P.R. was much less during the period April 3rd to 9th than during the other periods. This is perhaps due to the fact that he had spent several weeks in the country just before this period.

It has been suggested that the non-appearance of creatine in the urine (either as such or as creatinine) when given to adults is due to its destruction in the intestine by bacteria [Twort and Mellanby, 1913]. Our experiments do not support this view. In the case of amyotonia congenita the increased excretion of creatine after its administration was apparent so early that we are forced to believe that the creatine was rapidly absorbed, which agrees with the experiments of Folin and Denis [1912, 2] on cats. Further, the administration to the boy F.P.R. in the morning took place about half an hour before breakfast when the intestines may be considered to have been empty and therefore bacterial action on the creatine favoured. In the evening it was administered about 1½ hours after the last meal and would therefore be passed into intestines containing food, conditions unfavourable to the bacterial degradation of creatine. Yet in the former case there was increased excretion, but not in the latter.

DISCUSSION.

The results so far obtained with regard to creatinuria in children are most simply explained by assuming that this is due to the immaturity of the muscles. Creatine is not entirely an index of endogenous metabolism in the way that creatinine is, because the two do not show parallel variations. This may be due to the fact that creatine is utilisable, whereas creatinine is not. The production of creatine at night is probably lower than during the day, and anabolic processes of rest, by utilising or destroying part of it, cause a further diminution in the amount excreted. Sleep is apparently not the only factor involved, since the creatine excretion during the afternoon is less than during the morning, especially when reckoned as percentage of the "total creatinine." Assimilation of food has apparently little or no influence, since taking 130 cc. of milk at 8 p.m. did not influence the creatine excretion during the ensuing night. The chief factor appears to be the state of rest of the skeletal muscles associated with the temporary cessation of voluntary control which occurs during sleep.

The fully matured muscle produces only creatinine, and no creatine, or only such small amounts that utilisation processes prevent their coming to excretion. As the child develops, the creatinine coefficient gradually increases, and the creatine diminishes until it finally disappears. An extreme case of immaturity is seen in amyotonia congenita, in which the muscles are soft, toneless, and under-developed. Accompanying this condition, the excretion of creatinine per kilo. body-weight was much lower, and that of creatine much higher, than for a normal child of the same age.

SUMMARY.

(1) Suggestions are made improving the new Folin method of estimating creatinine in the case of urine very dilute in respect to this, and observations are recorded which support the accuracy of the new Folin method of creatine estimation.

(2) The diurnal and nocturnal variations in the excretion of creatinine and creatine have been determined for a normal boy, aged 2½ to 3 years.

(3) The rate of creatinine excretion is slightly less during the night than during the day.

(4) The rate of creatine excretion is highest during the morning, less during the later part of the daytime, and is very small during the night.

(5) If 0·1 g. creatine is given at the beginning of the morning most of it is excreted as such in the urine during the ensuing 12 hours, but if the same amount is given just before going to bed, there is no increase in the creatine excretion.

(6) The percentage of the total nitrogen in the urine which is present as ammonia is smaller during the day than during the night, showing that the higher creatine excretion during the day is not due to greater acidosis.

We have much pleasure in acknowledging that the expenses of this investigation have been defrayed by the Medical Research Committee.

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XXXI. NOTE ON SOME EXPERIMENTS WITH GLYOXALINE DERIVATIVES.

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(*From the Department of Biochemistry and Pharmacology of the Medical Research Committee.*)

(*Received August 7th*, 1916.)

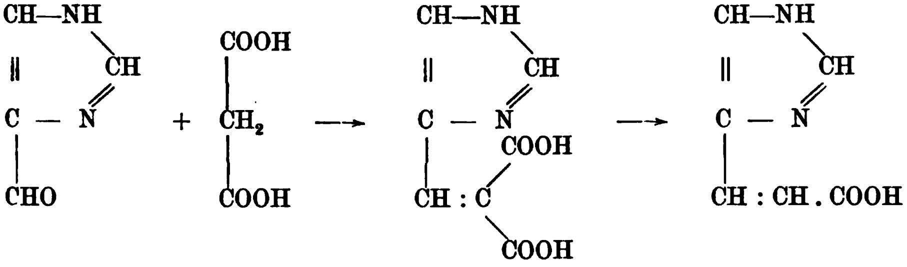
This note deals (1) with the behaviour of glyoxaline-formaldehyde in the organism of the dog, (2) with the condensation of glyoxaline-formaldehyde and malonic acid, (3) with the action of sulphuric acid on α-hydroxy-β-glyoxaline-propionic acid.

I. Furfuraldehyde, when injected hypodermically into the dog, was shown by Jaffé and Cohn [1887] to be changed principally into pyromucic acid and the condensation production of this acid with glycine, namely pyromycuric acid, but to the extent of a few per cent.; there is also produced furfurylacryluric acid, which is formed from glycine and furfurylacrylic acid. This latter observation induced us to administer glyoxaline-formaldehyde to a dog, in the hope that it might similarly be converted into urocanic acid, identified by Hunter [1912] as glyoxalineacrylic acid, which in two dogs has been observed as an abnormal, yet constant product of metabolism.

Accordingly 2 g. of glyoxaline-formaldehyde, for which we are indebted to the kindness of Dr F. L. Pyman [1916], was given hypodermically in 40 cc. of water to a small terrier bitch. The 280 cc. of urine, collected in the first 24 hours, was evaporated in vacuo and the residue was extracted with methyl alcohol, which removed the whole of the substances giving Pauly's reaction. After evaporation of the methyl alcohol, the residue was dissolved in 200 cc. of 5 % sulphuric acid and precipitated with 90 g. of phosphotungstic acid (a slight excess); the precipitate, filtered off next day, dissolved almost completely in 280 cc. of boiling water, and on cooling a large part remained in solution. From the phosphotungstate insoluble in cold water no base could be isolated, but the solution yielded glyoxalinecarboxylic acid, as follows. The bases, after recovery by means of barium hydroxide and evaporation to dryness, were almost completely soluble in methyl alcohol, which left on evaporation a residue consisting largely of urea, but also giving a strong Pauly reaction. Most of the urea was removed by adding dilute nitric acid and the filtrate was neutralised and evaporated. The residue, dissolved in a little hot dilute alcohol, deposited a small quantity of a glyoxaline derivative which on recrystallisation from a mixture of methyl alcohol and acetone formed thin needles, closely resembling glyoxaline-carboxylic acid. A crystal of the acid obtained from the dog grew in a solution of the synthetic acid. The acid from the dog's urine melted at 272–273°, another specimen, recrystallised from water, at 279°, the synthetic acid at 275–276°, the mixture of the two at 276°. (The melting point depends greatly on the rate of heating; in the above cases the bath was previously heated to 260°.) The acid from the dog's urine on melting yielded a distillate which crystallised on cooling and then had the melting point 86–87°. (Glyoxaline melts at 88°.)

Micro-Kjeldahl. 0·0040 g. gave 4·95 cc. N/70 NH_3. N, 24·75 %. Calculated for $C_4H_4O_2N_2$: N, 25·0 %. This formation of glyoxalinecarboxylic acid from its aldehyde is therefore analogous to the production of pyromucic acid, observed by Jaffé and Cohn, but the yield was very small and no trace of urocanic acid could be detected, perhaps because the glyoxaline ring is broken down too readily by oxidation.

II. It was thought that urocanic acid might be formed *in vitro* from the aldehyde and malonic acid, with subsequent removal of carbon dioxide.



I II III

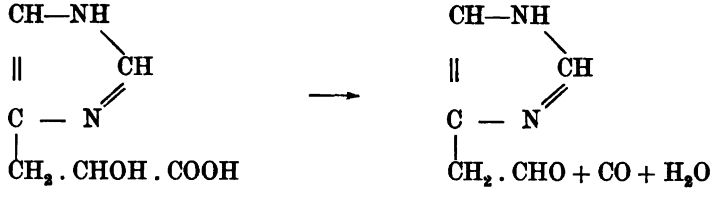
The substance II (*glyoxaline-methylidene-malonic acid*) is readily produced but so far it has been impossible to transform it into urocanic acid (III).

Knoevenagel [1904] showed that a variety of aldehydes readily condense with malonic acid and allied substances, when the reaction is catalysed by the addition of ammonia or a primary or secondary amine. The base is supposed to unite with the aldehyde giving a product which reacts readily with the malonic acid. In the present case it is interesting to note that the basic glyoxaline-formaldehyde readily condenses with malonic acid without addition of a catalyst, on simply warming the two substances in aqueous solution on the water-bath. The aldehyde (1 g.), malonic acid (1·2 g.) and water (25 cc.) were heated together. A practically clear solution resulted from which almost at once the condensation product (1·6 g.) began to separate as a finely crystalline meal. The substance is sparingly soluble in water or acetic acid, and very slightly soluble in alcohol or ether. It crystallises from water in prisms, which melt at 214° with decomposition and evolution of much gas. It gives a finely crystalline sparingly soluble *nitrate*, thus resembling urocanic acid. The substance is strongly acidic and may be fairly accurately titrated with baryta and phenolphthalein, the acid neutralising two equivalents of alkali. Permanganate is readily decolorised in both alkaline and acid solution.

Micro-Kjeldahl. 0·0128 g. gave 9·65 cc. N/70 NH_3. N, 15·1 %. Calculated for $C_7H_6O_4N_2$: N, 15·4%.

All efforts to remove one molecule of carbon dioxide from the substance, so as to yield urocanic acid, were unsuccessful. The substance was heated directly with and without glycerol to 200°, with water in a sealed tube to 150°, with acids and with alkalies. Carbon dioxide was in many cases readily removed, but urocanic acid could not be isolated from the products of the reaction. With water at 150° the reaction product was very soluble in water and could not be crystallised.

III. With a view to obtaining glyoxaline-acetaldehyde in the same way that acetaldehyde is formed from lactic acid, α-hydroxy-β-glyoxaline-propionic acid was heated with sulphuric acid, and a molecular proportion of carbon monoxide was indeed eliminated. The primary reaction is no doubt expressed as follows:



but then the aldehyde undergoes the further loss of a molecule of water and two molecules are condensed, the whole reaction being represented as follows:

$$2C_6H_8O_3N_2 = C_{10}H_8N_4 + 2CO + 4H_2O.$$

The constitution of the base $C_{10}H_8N_4$, containing two glyoxaline rings, has not yet been established, but it is evidently closely analogous to the hydrocarbon $C_{16}H_{12}$ obtained by Breuer and Zincke [1878] by heating phenylacetaldehyde and allied compounds with sulphuric acid, diluted with an equal volume of water. The hydroxy-glyoxaline-propionic acid was prepared from histidine by Fränkel's method [1903] and the substance containing a molecule of water of crystallisation was heated with three times its weight of sulphuric acid. With acid more dilute than 75 % no carbon monoxide is evolved until the acid has been concentrated. With 85 % acid evolution is slow at 150°, but complete at 170–180°. With 90 % acid carbon monoxide begins to appear at 125° and is liberated completely at 150–160°. With acid of a still higher concentration charring is apt to occur. The best concentration is therefore 85–90 % of H_2SO_4 and the yield of carbon monoxide approaches closely to the theoretical.

0·312 g. of hydrated α-hydroxy-β-glyoxaline-propionic acid (= 1/50 gram-molecule) was heated with 1 cc. of 85 % sulphuric acid in a test tube in a metal bath. Previous experiments had shown that no trace of carbon dioxide is formed in the reaction, so the air in the test tube was swept out by means of a current of carbon dioxide and subsequently the monoxide formed was collected in the same way in a Schiff nitrometer. The gas evolved measured 48·0 cc. at 20° and 760 mm., of this 0·2 cc. was absorbed by alkaline pyrogallol and 46·6 cc. by ammoniacal cuprous chloride, leaving a residue of 1·2 cc. The oxygen and the residue, probably nitrogen, were doubtless introduced as air from the Kipp apparatus.

46·6 cc. CO (moist) at 20° and 760 mm. = 0·052 g. or 93 % of the theoretical.

At the end of the experiment the sulphuric acid solution was pale brown and strongly fluorescent; the fluorescence is due to a substance hardly soluble in hot water, but readily in alcohol, which could not be obtained pure. The sulphuric acid may after dilution be neutralised with barium carbonate and after filtering hot, the base $C_{10}H_8N_4$ crystallises from the filtrate, or it may be obtained more simply by the addition of concentrated ammonium hydroxide, which at once precipitates it; yield 55–68 % of the theoretical. The deficiency is due to the formation of the fluorescent substance and of a substance readily soluble in water but not in alcohol, which can be obtained from the final mother liquor after quantitative removal of the sulphuric acid by barium hydroxide. This second substance is probably glyoxalinecarboxylic acid; it crystallised from water in thin needles, closely resembling the synthetic acid, and melted at the same temperature (284°).

Properties of the base $C_{10}H_8N_4$.

The base is readily soluble in cold alcohol, but only slightly in hot water (about 1 %), from which it is best recrystallised by addition of charcoal. In the crude state it forms small characteristic lozenge- or diamond-shaped plates, with rounded corners, but after recrystallisation irregular leaflets are obtained, which may be a centimetre long but still tenaciously retain a little colouring matter; by sublimation in the vacuum of a Gaede pump it is, however, obtained quite white. The substance crystallised from water or dilute alcohol has the composition $C_{10}H_8N_4$, H_2O. The water of crystallisation is given off in a vacuum over phosphorus pentoxide at 100°, but under atmospheric pressure only at a considerably higher temperature. The anhydrous substance is very hygroscopic, and the hydrated substance was therefore analysed, after drying in a steam-bath until constant.

0·1408 g.; 0·0635 g. H_2O and 0·03093 g. CO_2,
0·00965 g. (Micro-Kjeldahl) gave 13·4 cc. N/70 NH_3,
0·0592 heated to 150° in air-bath until constant lost 0·0053 g.
0·1041 heated over P_2O_5 at 10 mm. and 100° lost 0·0090 g.

The molecular weight was determined by the micro-method due to one of us [Barger, 1904].

0·0508 g. in 0·81 g. ethyl alcohol was equivalent to 0·325 g. β-naphthol, whence M.W. = 193.

	C	H	N	H_2O	M.W.
Found	59·9	5·0	27·8	8·7, 8·9	193
Calc. for $C_{10}H_8N_4$, H_2O	59·4	5·0	27·7	8·9	202

Heated to 150° the hydrated substance sinters without melting; plunged suddenly into a bath at 160° it melts, but gradually solidifies again to the anhydrous substance, which melts at 235–238°. In a Gaede pump vacuum it sublimes with metal bath at 260°. It has not been possible to crystallise the anhydrous substance from non-aqueous solvents although the original crystalline form is preserved when the substance is dehydrated at 100° in a vacuum; exposed to the air, the anhydrous substance rapidly takes up rather more than a molecule of water.

The substance $C_{10}H_8N_4$ is a base, readily soluble in hydrochloric or acetic acid. The *nitrate* is, however, very slightly soluble and crystallises well from water; the *picrate* is hardly soluble in boiling water. The base has practically no effect on the blood pressure or at most, exerts a very slight pressor action.

Action of other reagents on α-hydroxy-β-glyoxaline-propionic acid.

By heating the above acid with baryta to 205°, Fränkel [1903] obtained a substance, very slightly soluble in water and melting at 216°, in quantity insufficient for analysis. We repeated this experiment on a much smaller scale but could not isolate Fränkel's substance nor the base $C_{10}H_8N_4$ and therefore cannot draw any conclusion as to their possible identity.

With syrupy phosphoric acid at 200° no reaction occurs. At 220–240° about one molecule of carbon monoxide is given off and the solution becomes intensely fluorescent; after removal of phosphoric acid by baryta and evaporation, the residue was found to consist, for the smaller part, of the fluorescent substance, slightly soluble in cold water, readily in alcohol, and for the larger part of a substance readily soluble in water but insoluble in alcohol. The second substance on heating with 85 % sulphuric acid to 180° is transformed into the crystalline base $C_{10}H_8N_4$.

Concentrated hydrochloric acid at 235° acts in much the same way as phosphoric acid, yielding a fluorescent mixture of substances.

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XXXII. FEEDING EXPERIMENTS WITH A DIETARY IN WHICH TYROSINE IS REDUCED TO A MINIMUM.

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Knoop's discovery [1910] that the α-amino-acids can be synthesised in vivo from non-nitrogenous organic compounds and ammonia is one of the most significant contributions to the physiology of metabolism. He and Kertess [1911] isolated the *d*-acetyl derivative of γ-phenyl-α-aminobutyric acid from the urine of a dog to which the sodium salt of the corresponding α-ketonic acid had been given subcutaneously, demonstrating the fact that the synthesis of the amino-acids from ketonic acids can be effected in the animal body.

As a result of the administration of γ-phenyl-α-hydroxybutyric acid to a dog, there was a rise in the excretion of the corresponding amino-acid, showing that the α-hydroxy-acids can be converted into the α-amino-acids.

Applying the method of liver perfusion to the problem, Emden and Schmitz [1910, 1912] have demonstrated that an analogous process may take place in connection with the synthesis of the normal protein constituents, such as tyrosine, phenylalanine and alanine from the corresponding ketonic acids, thus showing that the phenomenon of amino-acid synthesis is a general reaction.

The well-known observations of Loewi [1902] followed by the elaborate experiments of Abderhalden, Henriques and others, have conclusively proved that an animal can maintain itself and exhibit growth when free amino-acids replace the protein of the food.

It was the original intention of the present research to decide whether tyrosine can be replaced by its corresponding ketonic acid in a dietary in which the mixture of the hydrolysis products of caseinogen were the sole source of nitrogen supply; it being assumed from the work of Abderhalden [1913] that, with a deficiency of this aromatic amino-acid, an animal is unable to exhibit normal nutrition. My preliminary experiments led, however, to the unexpected result that in the case of rats most of the individuals fed upon a dietary which contained at most a minimal supply of tyrosine, not only maintained their body weight, but also exhibited growth. The original intention of my research could not therefore be carried out, but the experiments which show the apparently unessential nature of tyrosine seem to be of interest, and are discussed in this paper.

ON THE QUANTITATIVE ISOLATION OF TYROSINE FROM CASEINOGEN.

Before attempting the feeding experiments, an endeavour was made to isolate tyrosine quantitatively from the hydrolysis products of protein. By applying the method of recrystallisation to the amino-acid mixture from caseinogen hydrolysed by acid, in spite of adopting the details of the method of Abderhalden and Fuchs [1913], it was found to be extremely difficult to get rid of all substance yielding the Millon's reaction from the last filtrate, though no more crops of the characteristic crystals of tyrosine could be obtained therefrom by further crystallisation. In three experiments, in two of which hydrolysis was effected with sulphuric acid and in one with hydrochloric acid, the amounts of tyrosine obtained were respectively 3·45, 3·70 and 3·23 %. In comparison with the figure 4·5 %, which is provisionally accepted as the probably correct proportion of tyrosine in caseinogen, the above figures are evidently low, but, on the other hand, they are closely similar to those obtained by Osborne and Guest [1911], viz. 3·9, 3·2 and 3·4 %. These latter authors, in spite of careful work, were also unable to isolate the whole of the tyrosine present in caseinogen by direct crystallisation. It is evident that the solubility of tyrosine is profoundly affected by other amino-acids present in the mixture. In the hope of eliminating this influence, the following method was used. Caseinogen was boiled with sulphuric acid of 25 % strength for 72 hours, so that complete hydrolysis might be effected. After the removal of sulphuric acid, the solution was submitted to repeated crystallisation, the process of evaporation being always performed in vacuo. Even when the tyrosine had been separated as completely as possible, the filtrates still gave a Millon's reaction with apparently undiminished intensity. Starting with 30 g. of caseinogen, 1·02 g. of tyrosine were obtained in characteristic needles. To the filtrate an aqueous solution of mercuric chloride was added; the first portions of the precipitate thus formed contained histidine [cf. Kossel, 1896], which was identified by means of its picrolonate. On the further addition of the precipitant to the now distinctly acid solution, more precipitate was obtained, which partly consisted of a sulphur-containing compound, probably cystine [cf. Suter, 1895]. These precipitates were filtered off, and the filtrate was so far saturated with mercuric chloride that the solution contained sufficient to give a yellow precipitate with baryta water; then, finely powdered baryta was added with continuous stirring until tyrosine was completely precipitated, as shown by the absence from the filtrate of Millon's reaction and of the modified diazo test for tyrosine [Totani, 1915]. Excess of baryta should be carefully avoided, as the tyrosine compound is apt to be redissolved in excess. In several experiments it was found that 50 g. of baryta were sufficient to precipitate all the tyrosine present in a litre of the solution. The precipitates were filtered off by suction and washed with baryta water; they were then suspended in water containing a slight excess of sulphuric acid, and decomposed with hydrogen sulphide. The filtrate and washings from the mercury sulphide and barium sulphate were concentrated; chlorine removed by means of silver sulphate, and the excess of silver by hydrogen sulphide. The whole solution was now made up to 500 cc. and contained approximately 5 % sulphuric acid. It was then precipitated with 10 % phosphotungstic acid solution. After leaving overnight, the precipitate was filtered off, and washed with 5 % sulphuric acid, until the washings no longer yielded Millon's reaction. The filtrate and washings were freed from an excess of phosphotungstic acid by baryta and subsequently from the latter by sulphuric acid. They were then submitted to repeated crystallisation. In this manner a further quantity of 0·23 g. of pure tyrosine was obtained. The amino-nitrogen content of this sample was estimated by means of van Slyke's method:

0·0554 g. gave 7·8 cc. N at 19° and 758 mm. = 8·03 % N.

Calculated for $C_9H_{11}O_3N$	Found
N 7·73 %	8·03 %

The tyrosine was therefore probably pure.

Since the filtrate from the last crystallisation still gave an intense Millon's reaction, an endeavour was made to crystallise more tyrosine, but without success. The total quantity of tyrosine isolated was 4·16 % of the protein used; considerably more than that obtained by direct crystallisation.

For the purpose of isolating tyrosine, therefore, it is advantageous to remove other constituents as completely as possible from the hydrolysis mixture. Based on the observation that the more soluble fractions of a hydrolysis mixture will retain tenaciously material yielding a Millon's reaction, Gortner [1911] drew attention to the possible presence of some other aromatic phenolic body in the molecule of keratin. Guggenheim [1913] isolated dihydroxyphenylalanine from *Vicia fava*. This reacts with Millon's reagent, and it is possible that such a compound may exist in the protein molecule.

The above method of isolating tyrosine was beyond doubt more nearly quantitative than direct crystallisation, but it proved to be extremely tedious and unsuitable for preparing material for feeding experiments.

The results of numerous experiments agree in showing that tyrosine is liberated almost completely at a very early stage of tryptic digestion. Abderhalden and Voegtlin [1907] isolated 4·5 % from the tryptic products of caseinogen.

Advantage was taken of the above fact in a further endeavour to separate tyrosine with a greater completeness. Caseinogen was first digested with trypsin, and the liberated tyrosine filtered off. The filtrate was then submitted to acid hydrolysis in the hope of obtaining a further yield.

The tyrosine which separated from a thorough pancreatic digest of 60 g. of caseinogen was filtered off and purified by recrystallisation from water. The filtrate was concentrated on the water-bath and kept cool in a freezing mixture for several hours, and a further quantity of pure tyrosine obtained. Submitting the main solution to further crystallisation, it was found that a little more tyrosine could be isolated, but only after a non-Millon-yielding fraction, probably leucine, was first removed. The amount of tyrosine thus obtained was 2·27 g.

The whole solution was now made up to 400 cc., with the addition of sulphuric acid to the extent of 25 %, and boiled under a reflux condenser for 72 hours. After removal of the sulphuric acid, the solution was submitted to crystallisation until no more tyrosine could be isolated. The quantity of tyrosine isolated as the result of the secondary acid hydrolysis was 0·174 g.; the whole yield obtained amounting therefore to 4·07 % of the protein. In another analysis carried out with 100 g. of caseinogen, 4·02 g. of tyrosine were obtained.

In connection with the above analysis, the following bromination experiments were carried out. Millar [1903] first worked out a method for estimating tyrosine by measuring the quantity of bromine necessary to form the dibromo-substitution compound. Plimmer and Eaves [1913] modified the method so that the estimation of even small amounts of tyrosine could be effected with accuracy; they applied the method in an endeavour to determine the tyrosine content of protein. It was found, however, that the presence of histidine and tryptophane, both capable of absorbing bromine, was a disturbing factor. The former could be removed by phosphotungstic acid, but, with regard to tryptophane, it was found that although this amino-acid was destroyed by boiling with acid, its decomposition products still reacted with bromine; accordingly, tyrosine could not be estimated by the bromine method in a solution containing the whole of the products of acid hydrolysis. Plimmer and Eaves therefore utilised a tryptic digest of the protein in which the tyrosine had been liberated at an early stage of the hydrolysis, while tryptophane was assumed to be still in combination.

It is clear that the bromination method cannot be applied for the purpose of determining how much tyrosine remains in solution after its isolation by direct crystallisation has been attempted. The amount of tyrosine separated may be measured, however, by determining the bromine absorbed by the whole hydrolysis mixture, and that absorbed after the tyrosine has been crystallised out; the difference should correspond to the amount of tyrosine obtained. This method I have applied. In a preliminary experiment the quantity of bromine absorbed by pure tyrosine was determined; this gave 1·803 g. of bromine for 1 g. of tyrosine. The figure is rather higher than the calculated value, 1·765 g., but agrees with the results obtained by Millar and by Plimmer.

The solution of hydrolysis products from 60 g. of caseinogen freed as completely as possible from tyrosine in the former experiment (solution I) was submitted to bromination. As control material the same quantity of caseinogen was treated in a precisely similar manner, but the tyrosine not separated (solution II). Each solution was made up to 500 cc. with the addition of a small quantity of hydrochloric acid to complete the solution of the amino-acids. 25 cc. of each solution were brominated.

	Bromine absorbed (in g.) by	
	Solution I	Solution II
	0·4200	0·6349
	0·4176	0·6334
Mean	0·4188	0·6341

Difference = 0·2153 for 3 g. protein,

∴ 7·176 for 100 g. protein.

Since 1 g. of tyrosine absorbs 1·803 g. of bromine, 7·176 g. correspond to 3·98 g. of tyrosine. From the solution I, tyrosine was, as previously stated, isolated to the extent of 4·07 % by the crystallisation method; the figures obtained agreed, therefore, within the limits of experimental error. It is clear, therefore, that tyrosine was removed from the amino-acid mixture to the extent of 4 % of the protein used. No attempt to estimate with accuracy the amount of tyrosine left in solution was successful.

Feeding Experiments with Caseinogen.

Method. All the experiments described in what follows were carried out on the lines adopted by Hopkins [1912] in his investigations dealing with the accessory growth factors. Young albino rats were employed; they were taken from a stock which had been fed upon oats and bread and milk. The origin and previous history of the animals were known. The experiments were carried out on strictly comparative lines. The experimental and control animals were so chosen that the groups compared were uniform in origin, sex and weight. They were fed and treated in an exactly similar manner, except with regard to the essential factor with which the investigation was concerned. Rats of the same sex were kept two together, bucks with bucks and does with does, in wire cages which were placed in a cellar of uniform temperature. The food was given in excess of consumption, and water was administered freely. Having regard to the results obtained by Hopkins and others, it is clear that when synthetic diets are fed, it is necessary to supply the accessory growth factors. In my experiments these were administered in the form of an alcoholic extract of milk, in a quantity such that the two rats received an amount corresponding with 1·5 cc. of fresh milk per diem. Observations were made on the change of weight and on the general well-being of the animals. In one experiment the survival period was determined. Nitrogen equilibrium was not determined. Henriques [1909] observed that the body weight of a rat might be maintained for some days, even when nitrogenous equilibrium no longer existed. Experiments carried out in this laboratory show, however, that such a relation is only temporary. The rats were weighed daily at a specified time, and the amount of food consumed during the previous 24 hours determined.

Experiment I.

In this the sole nitrogenous supply to the animal was an amino-acid mixture from which the tyrosine had been removed as completely as possible on the lines discussed in previous sections.

I have shown above that tyrosine can be more completely separated by making use of a combined tryptic and acid hydrolysis, followed by repeated crystallisation, than by the usual method. From 1 kilogram of the commercial caseinogen preparation known as "protene" 38·52 g. of tryosine were obtained in an apparently pure state. This figure is lower, of course, than that obtained as above from pure caseinogen, but making allowances for the impurities in "protene," the separation is, as a matter of fact, equally complete. The amino-acid mixture from which the tyrosine had been thus removed was used in preparing the diet for the experiment now to be described. The solution was evaporated nearly to dryness, and then a known quantity of potato starch was added. The whole mass was so far dried that the material could be easily powdered by means of a coffee mill. Fat and cane sugar were subsequently mixed in, and tryptophane to the extent of about 1·5 % of the protein which yielded the amino-acid mixture.

In the control diet, tyrosine was added to the extent of 3 % of the original protein.

As regards nitrogen content, the food was prepared in two forms: one containing 3 and the other 2 %. The actual composition of the diets was as follows:

	Food of 3 % N content	Food of 2 % N content
Products from hydrolysed protein and starch mixed (N 7·0 %)	43·0 %	28·6 %
Tryptophane	0·5	0·4
Starch	20·1	34·6
Cane sugar	21·2	21·2
Fat	12·5	12·5
Salts (ashes of oats and dog biscuit in equal proportion) ...	2·7	2·7

Before feeding, a small quantity of water was added, and rubbed in by hand, so that the food mixture was obtained in the form of lumps of adequate size and consistence for consumption by the rats. The dried alcoholic extract of milk was administered in a small daily ration as mentioned above.

Eight healthy male animals were employed: of these four were fed upon the basal diet only (set A), the other four received the tyrosine addition (set B). During the first 24 days all received the food containing 3 % of nitrogen; later, that containing 2 % was given with the intention of diminishing the tyrosine supply.

The result of the experiment may be summarised in the following figures:

Set	Rat No.	Duration of feeding on 3 % N food in days	Gain in weight in %	Duration of successive feeding on 2 % N food in days		Further gain in weight in %	
				First	Following	First	Following
A	1	24	38·6	10	7	0	7·5
	2	24	36·6	10	7	5·0	5·5
	3	24	34·6	10	—	9·5	—
	4	24	22·1	10	—	9·7	—
			Mean 32·9			6·0	
B	5	24	26·0	10	—	9·9	—
	6	24	30·9	10	—	7·4	—
	7	24	37·0	10	—	4·2	—
	8	24	26·1	10	—	8·5	—
			Mean 30·1			7·5	

The behaviour of individual animals was closely similar in all cases, and no marked difference between the two sets could be established. All the animals consumed the food equally well, and remained in good condition. Wet filter paper was given from time to time as a precaution, but no intestinal trouble was any time observed. From this experiment it may be concluded that rats may exhibit almost normal growth when the supply of tyrosine is diminished to an extremely small amount. This result, however, differs from that obtained by Abderhalden. In one of his experiments [Abderhalden, 1913] a dog was used; a preparation of the digestive products of caseinogen freed from tyrosine as completely as possible, by direct crystallisation alone, was given to the animal, which lost body weight to the extent of 750 g. in nine days. There was a further loss in the four following days, though this was partly caused by an insufficient intake of food. The loss of weight, however, was regained almost entirely when tyrosine was added to the previous dietary. Abderhalden therefore came to the conclusion that tyrosine is an essential factor in nutrition. As already mentioned, the original intention of the present research was to discover if tyrosine could be replaced in nutrition by the corresponding ketonic acid. When the work began there was, to the best of my belief, no suggestion in the literature for the plan of testing the possible nature of intermediary metabolic products by replacement of the original amino-acid in a diet. Since then, however, in the third edition of his Lehrbuch, Abderhalden [1915, 1] has made brief mention of an experiment carried out on rats by himself for the purpose of investigating the replaceability of tyrosine by its ketonic acid. In this experiment the quantitative separation of tyrosine was unsuccessful; the rat, however, was unable to maintain its nitrogenous equilibrium on the reduced supply of tyrosine. The description of this research, mentioned incidentally in the text-book, is so brief that the details are not clear. It is obvious, at any rate, that Abderhalden's results were entirely different from mine[1].

It is, of course, an objection to my experiments that the actual amount of tyrosine left in the diet is uncertain. Abderhalden claims that in his amino-acid mixture not more than 0·2 % of tyrosine was left behind. It will be understood from what has gone before that to give an actual figure for this is impossible. It is extremely improbable, however, having regard to the methods that I used, that there was more tyrosine in the dietary used by me than in that employed by Abderhalden.

In the endeavour to avoid this difficulty, further experiments were carried out on a gelatin diet. In connection with the effect of tyrosine deficiency, it is important to remember the clear evidence obtained by Emden and Baldes [1913] that phenylalanine can be converted into tyrosine in the body. This was shown conclusively during the perfusion of the surviving liver with the former substance. An explanation, therefore, for the normal growth of animals with tyrosine deficiency may be sought in this conversion of phenylalanine, which is present in a considerable amount in protein, into the missing phenolic amino-acid. Such a rôle of phenylalanine in replacing tyrosine was suggested in a research of Abderhalden's [1914].

FEEDING EXPERIMENTS WITH GELATIN.

It has become so clear from recent researches that the efficiency of a dietary is not determined by the amount, but rather by the quality, of its nitrogenous constituents, that earlier researches upon the protein-sparing power of gelatin need not be referred to. So far back as 1876 Escher observed an improvement in the nutritive value of gelatin by the addition of tyrosine alone; but now that it is known that tryptophane, which is entirely absent from gelatin, is an essential factor for maintenance, any investigation made without the addition of this need not be considered. Kaufmann's [1905] paper is the first that needs attention. He found that protein could be replaced by gelatin, when tyrosine and tryptophane were added, to the extent of from one-third to one-half of the total nitrogen of the diet. Under these circumstances, the nitrogen balance was maintained. This experiment was carried out on a dog. In a further experiment performed upon himself, he reached the striking result that there was nitrogenous equilibrium when gelatin, with the addition of the absent amino-acids, replaced the whole of the protein of the diet. Kaufmann's experiment was repeated by Rona and Müller [1906] upon dogs. They could replace the protein by gelatin plus tryptophane and tyrosine, up to the extent of two-fifths of the total nitrogen, but not beyond this. They concluded, therefore, that the protein-sparing power of gelatin could not be enhanced by the addition of the missing aromatic amino-acid. Later on the problem was again attacked by Abderhalden and Manolin [1910], whose experiments confirmed Kaufmann's result. Because of the possible indigestibility of normal gelatin, these last observers employed predigested gelatin, to which several amino-acids, comprising not only those actually missing, but also those which are present in relatively small amount in the gelatin molecule, were added. In the dog, they found that this mixture could replace from three-fifths to two-thirds of the protein nitrogen in the food. Moreover, in a still later experiment, Abderhalden [1912] claims to have maintained a dog practically in nitrogenous equilibrium even on complete replacement of protein by the above mixture of gelatin and amino-acids. As a result of this experiment, Abderhalden assumes that a supply of the aromatic amino-acids is essential to life. As a matter of fact, however, until Abderhalden made the further reference in the text-book as mentioned above, to his study of tyrosine deficiency, there was really no evidence for the indispensability of an aromatic grouping, except in the case of tryptophane.

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[1] Abderhalden's full publication (1915, 2) was not seen until after this paper was written.

If a diet free from tyrosine is to be prepared from gelatin, it is essential that the gelatin should be pure. Among the many samples tested by me, the "gold label" gelatin of the French firm Coignet Père & Fils & Cie, with the mark "extra" was found to be practically free from other protein. Millon's reaction, however, was fairly distinct, when the gelatin was dissolved in water, with the addition of a little sulphuric acid, and excess of the reagent carefully avoided. Plimmer and Eaves [1913] submitted a gelatin[1] digest, previously freed from histidine, to bromination, and found that a certain amount of bromine was absorbed. They considered this due to the presence of tyrosine, and calculated its amount as 0·24 %. Recently, however, Siegfried and Reppin [1915] observed a marked difference in the amounts of halogen absorbed by gelatin according to the conditions under which bromination was performed. Intact gelatin absorbed much more than hydrolysed. They held that in the gelatin molecule some complex capable of combining with bromine might be present, which disappeared during acid hydrolysis. If this evidence be taken into consideration, the bromine value of gelatin, as found by Plimmer and Eaves, ought not to be regarded as due exclusively to tyrosine. Mörner [1899] states that even the purest gelatin gives a marked colour with Millon's reagent. Murlin [1907] endeavoured to purify gelatin by Kirchmann's method, but Millon's reaction remained undiminished in his final product. It is clear that at present we have no knowledge as to the nature of the Millon-yielding substance in gelatin. I had to be content, therefore, to use gelatin which, while certainly free from other protein, still yielded at least a slight colour with Millon's reagent. I shall now describe two feeding experiments, in one of which intact gelatin was used, and in the other the hydrolysed product.

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[1] Plimmer and Eaves used also "gold label" gelatin as material, but did not mention the variety.

Experiment (II) with non-hydrolysed gelatin.

A basal dietary was prepared in the following way. A jelly composed of 250 g. of pure gelatin and 420 g. of starch was dried to such a consistence that it could be passed through a mincing machine. This treatment was repeated until the whole could be sifted through a sieve of 1·5 mm. mesh, and the product was then completely dried. The other constituents, fat, sugar and salts, were finally mixed in the same proportion as in the diet of the previous experiment. The total nitrogen content of the basal dietary so prepared was 3·4 %. The calculated energy value was just over 5 calories per gram. The protein-free milk extract was given in a small ration as before. Three sets of animals were compared: the first set (C), were fed on the basal dietary alone; the second (D) received in addition tryptophane to the extent of 1·5 % of the gelatin used; the third set (E) were given, in addition to the tryptophane, tyrosine to the extent of 3 % of the gelatin used. The food was consumed equally well by all the rats, until a few days before their death. In all the sets, a steady loss of weight occurred from the beginning of the special feeding. The experiment was continued till all the animals had succumbed.

If set C be compared with set D, it will be seen that there was no difference either in the rate of loss of weight, or in the survival period. Even in the case of the rats fed upon the gelatin plus tryptophane and tyrosine, the difference was but slight. An extension of the survival period by one week, is, I think, too short to be of much significance[1].

Set C N in food supplied by gelatin			Set D N in food supplied by gelatin + tryptophane			Set E N in food supplied by gelatin + tryptophane + tyrosine		
Rat No.	Survival period in days	Loss in weight in %	Rat No.	Survival period in days	Loss in weight in %	Rat No.	Survival period in days	Loss in weight in %
9	17	45·0	15	16	45·7	21	37	44·8
10	30	44·8	16	23	46·1	22	37	47·2
11	32	46·0	17	38	58·1	23	36	42·9
12	30	45·0	18	39	51·8	24	44	47·8
13	30	40·8	19	27	45·5	25	33	44·2
14	28	48·0	20	24	44·8	26	32	47·3
Mean	27·8	44		27·8	48·6		34·8	45·7

Average loss of weight 46·4 %.

As the following experiment shows, the failure to obtain any improvement by the addition of the missing amino-acids was not due to the lack of their significance in metabolism, but to the fact that the intact gelatin itself is very badly absorbed.

Experiment (III) with hydrolysed gelatin.

In this experiment, the products of the acid hydrolysis of gelatin, were employed as the source of nitrogen supply in the basal dietary. The food was prepared exactly as in the case of the caseinogen preparation of Experiment I. The total nitrogen content was brought to the same value as that of the non-hydrolysed gelatin diet of Experiment II.

Six sets of animals were compared; in each set four rats (two bucks and two does) were employed. The rats of the first set (F) were for comparison fed upon the non-hydrolysed gelatin diet, as used in Experiment II. All the other sets were put upon the hydrolysed gelatin food. The second set (G) had this basal diet alone; the third (H) the same with the addition of tryptophane; the fourth (I) with tryptophane and tyrosine. To the last two sets (J) and (K) histidine and cystine were given in addition to the above, in the hope of still further enhancing the nutritive power of the gelatin. Cystine, of course, is missing from gelatin, and histidine occurs in a very small amount only. In set (K) tyrosine was replaced by synthetic phenylalanine.

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[1] It may be mentioned that three of the rats from set E displayed peculiar symptoms some 12–24 hours before death. Paralysis began in the hind-legs, and extended rapidly over the trunk to the fore-legs, so that the animal could no longer move. Clonic and tonic spasms then followed and death occurred in a few hours. A post-mortem examination revealed nothing remarkable. As to the cause of these symptoms, I have no suggestion to offer, but it does not seem probable that they were accidental. They certainly did not suggest a tetanus infection.

The histidine was obtained from ox blood by the method of Knoop [1907], the hydrochloride obtained being neutralised in solution with sodium carbonate before mixing with the food. The cystine was obtained from hair; the phenylalanine was prepared by the method of E. Fischer, the product used melting at 263°. The amounts of tryptophane and tyrosine added were the same as in the previous experiment. Histidine and cystine were added to the extent of 2 and 1 % of the gelatin respectively. As regards phenylalanine, though it is known that the *d.l.* compound can be completely consumed in the organism of the dog [Schotten, 1883; Knoop, 1905], and also that it increases the homogentisic acid formation, as much as the active compound [Abderhalden, Bloch and Rona, 1907], yet having regard to the general evidence for the necessity of supplying the natural form of amino-acids, the racemic phenylalanine was employed in quantity double that of the tyrosine it was to replace.

Set F				Set G			
Rat No. and sex	Change in weight in %	Duration of feeding in days	Remarks	Rat No. and sex	Change in weight in %	Duration of feeding in days	Remarks
27 ♂	-34·4	31	alive	31 ♂	-18·2	31	alive
28 ♂	-44·4	26	dead	32 ♂	-19·6	31	„
29 ♀	-33·8	31	alive	33 ♀	-17·9	31	„
30 ♀	-35·3	31	„	34 ♀	-17·5	31	„
Mean	-36·9				-18·4		
Set H				Set I			
35 ♂	+ 8·5	31	alive	39 ♂	-17·1	31	alive
36 ♂	-15·0	31	„	40 ♂	- 8·4	31	„
37 ♀	+ 0·4	31	„	41 ♀	+ 6·4	31	„
38 ♀	- 8·4	31	„	42 ♀	+ 1·4	31	„
Set J				Set K			
43 ♂	-26·2	22	alive	47 ♂	+11·3	22	alive
44 ♂	+ 5·2	22	„	48 ♂	+ 5·8	22	„
45 ♀	+ 3·2	31	„	49 ♀	-14·6	31	„
46 ♀	- 0·9	31	„	50 ♀	- 0·9	31	„

If now set (F) be compared with set (G), it becomes very clear that the previously hydrolysed gelatin can be much more readily employed by the rat than the non-hydrolysed, since the loss of weight for the given period is reduced to a half. The difference does not depend upon the quantity of food consumed. The following figures which give the average consumption per 100 g. body weight show that closely similar amounts of food were eaten by the animals in both sets.

A somewhat diminished consumption in the later periods in the case of the set taking the non-hydrolysed gelatin, may be considered as the effect and not the cause of the loss of weight, which was much more rapid than in the case of the animals taking the hydrolysed gelatin [cf. Hopkins, 1912]. It seems clear that the intact gelatin is badly digested and absorbed by the rat, and as already claimed, this fact explains the failure to obtain good results upon the addition of the missing amino-acids in the previous experiment. The results in the case of set (H) were remarkable. The treatment of the animals was exactly similar to that of set (G), save that a little tryptophane was given. Of four rats, two were not only able to maintain their weight, but also exhibited some growth. The general condition of these animals also remained satisfactory. The condition of the other two rats of this set was also for a long time much better than that of rats receiving no tryptophane. These two animals, it is true, lost weight; but in such experiments a positive result is clearly of the greater significance. It may be concluded, therefore, that rats can at any rate maintain themselves upon the hydrolysed products of gelatin when tryptophane alone is added.

	Set F			Set G		
	Food* eaten by			Food* eaten by		
Duration of feeding	Rats 27 and 28	Rats 29 and 30	Mean of loss of weight	Rats 31 and 32	Rats 33 and 34	Mean of loss of weight
1—3 day	6·4 g.	7·9 g.	12·4 %	5·7 g.	5·5 g.	8·3 %
4—6 ,,	7·4	7·1		8·4	8·8	
7—9 ,,	8·3	7·6		7·9	8·9	
10—12 ,,	7·1	7·1		7·4	7·6	
	Mean 7·3 g.			7·5 g.		
13—15 day	7·1 g.	5·8 g.	22·2 %	8·4 g.	7·3 g.	4·9 %
16—18 ,,	6·7	5·8		7·7	7·3	
19—21 ,,	6·4	5·8		7·9	7·1	
22—24 ,,	7·2	6·2		7·8	6·4	
	Mean 6·4 g.			7·4 g.		

* The average daily food consumption per 100 g. weight during the experimental period.

In set (I) tyrosine was given in addition to tryptophane, with scarcely any effect. Sets (H) and (I) closely agree. If tyrosine improves the nutritive value of gelatin, its effect is certainly much less than that of tryptophane.

In the last two sets (J) and (K), which received histidine and cystine in addition to tryptophane and tyrosine (or phenylalanine), the condition of the rats was somewhat more satisfactory than in the other sets. I think this experiment may be taken as showing that the complete replacement of protein by hydrolysed gelatin plus the missing amino-acids is quite possible. If tyrosine be necessary for maintenance and growth, phenylalanine seems able to act as raw material for its formation.

DISCUSSION OF RESULTS.

Perhaps the most important point brought out in the above experiments is the fact that the rat can maintain itself and even gain weight on a hydrolysed gelatin diet, with the addition of the single amino-acid, tryptophane. Based upon the feeding experiments carried out with Miss Willcock, Hopkins [Willcock and Hopkins, 1906] has suggested that the protein constituents may be utilised not only for tissue formation, or structural maintenance, but in a more specific and direct manner, as, for instance, in the elaboration of essential substances such as adrenaline or other hormones. We have no direct evidence at present to show that adrenaline is derived from tyrosine, though the close resemblance in the chemical constitution of these two substances makes it very tempting to believe that the former is the precursor of the latter.

When animals are observed to grow normally upon food containing most minute quantities of tyrosine (Experiment I, set (A)), it is almost certainly because phenylalanine is capable of replacing tyrosine and serving as its precursor. The amount of phenylalanine in protein is sufficiently great to justify this belief. As regards the result of the experiment in which there was maintenance with tryptophane only added to the gelatin hydrolysis products, some further explanation seems necessary. In this case the total amount of the aromatic groups in the diet was very small. The minimal requirement of the animal in this respect is of course not yet known. It appears probable, however, that the amount of phenylalanine in the gelatin dietary is too small to cover the requirement, and one has to think of the possibility of the synthetic formation of the benzene ring in the body. Recent advances in the physiology of intermediary metabolism have suggested that the synthetic power of the body is much greater than was previously thought. With respect to the simpler aliphatic amino-acids, such as glycine and alanine, it may be taken as already proved that they can be built up in the animal body. As regards the synthetic formation of the more complex amino-acids, such as histidine and phenylalanine, proof is yet lacking. As is well known, Knoop and Windhaus [1905] have suggested that histidine may be formed in the organism from methyliminazole and glycine, by means of an oxidative synthesis, the former substance arising from glucose and ammonia. As regards the synthesis in the organism of amino-compounds derived from benzene or phenol there is, so far as I know, nothing yet known. Nevertheless, the above-mentioned fact that the animal can subsist without suffering tissue breakdown on hydrolysed gelatin and tryptophane alone, points, I think, to the possibility of a synthesis of a benzene nucleus. On the other hand the indole ring, as many experiments in this laboratory seem to show, cannot be dispensed with from the diet.

SUMMARY.

(1) Tyrosine could not be isolated quantitatively from the hydrolysis products of caseinogen.

(2) When the removal of tyrosine from the amino-acid mixture is made as complete as possible, and is effected to an extent which certainly leaves only minimal quantities of this constituent, there appears to be no effect upon the nutritive value of the amino-acid mixture.

(3) In the case of the rat, the nutritive efficiency of gelatin is greatly increased by previous hydrolysis. Gelatin when fed intact appears to be badly digested and absorbed.

(4) The possibility of completely replacing the protein of a diet by hydrolysed gelatin, plus certain amino-acids, is confirmed.

(5) Some evidence is offered that the addition of tryptophane alone to the hydrolysis products obtained from pure gelatin made these efficient in maintaining the nutrition of animals.

The subject of the present investigation was suggested to me by Professor F. G. Hopkins who is responsible for the feeding experiments and to whom I am greatly indebted for valuable advice.

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[1] Owing to the present circumstance, this book was only obtained at the end of October, 1915, when the present investigation was nearly finished.

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XXXIII. A GENERAL METHOD OF ESTIMATING THE RELATIVE TURBIDITY OR OPACITY OF FLUID SUSPENSIONS INCLUDING BACTERIAL EMULSIONS.

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Methods of estimating the turbidity or opacity of fluids have been employed by chemists for some years past. T. W. Richards [1895] was the first to describe an apparatus, the "nephelometer," designed for this purpose. Later P. A. Kober [1913] found it possible to estimate turbidity with a modified Duboscq colorimeter; and the same instrument has been employed in varying modifications by Marriott [1913], Folin and Dennis [1914], W. R. Bloor [1914] and others. Dreyer and Hansen [1907] made use of the turbidity, or rather the "clarification," of fluids in an investigation of the effects of light upon enzymes etc. They carried out their experiments in dwarf test-tubes, arranged in series to contain diminishing concentrations of the active substance in a constant volume of substrate, and they found that, when dealing with a turbid substrate, the extent of the reaction could be ascertained by reading the degree of clarification of the fluid. This procedure, although used for a quite different purpose, embodies the main features of the method described in this paper.

Measurements of the turbidity or opacity of fluid suspensions have hitherto lain almost exclusively within the province of pure or applied chemistry, and no great effort has been made to employ accurate methods of this kind in the service of bacteriology. Nevertheless there are numerous bacteriological procedures which would be facilitated by an accurately quantitative method of turbidity estimation; and for one process in particular it is absolutely essential to use a method of this kind. We refer to standardisation of agglutinable cultures. During the last twelve months the particular macroscopic method of agglutination, described by one of us [Dreyer, 1909] has come widely into use. The most essential feature of the method is the employment of sterile formalised and standardised bouillon cultures of the bacteria. The standardisation process is a double one, and concerns firstly the opacity or turbidity of the culture, and secondly its specific sensitiveness to agglutination.

The second part of the process does not concern us here [see Davison, 1916]. The measurement of the turbidity of the cultures is of great importance, because upon it depends the ease and accuracy with which their relative sensitiveness to agglutination can be estimated; for it is very difficult to compare a thin culture with a thick one, or *vice versâ*, whereas the sensitivity-estimation of cultures of equal thickness presents, as a rule, little difficulty. The turbidity-test does not necessarily provide an accurate measurement of the number of bacteria in a bouillon culture, for the total turbidity will include any initial opalescence of the bouillon in which the bacilli are grown. The difference in sensitiveness of the cultures is indicated by the sensitivity-constants of the individual cultures.

The first apparatus which we employed for turbidity-standardisation was the Duboscq colorimeter modified in a new way to serve our special purpose. Within certain limits this yielded very satisfactory results, but a serious obstacle to uniformity and certainty of the readings was encountered in the fact that our veal-bouillon cultures were not of uniform colour. The deeper the colour of the fluid, the lower were the readings for a given degree of turbidity. Various means of overcoming this difficulty suggested themselves, and of these one promised considerable success; viz. the addition of a strong deep-brown peptone solution to the paler of the two fluids to equalise the colours. But after a considerable number of these experiments had been carried out the method was discontinued and replaced by the much simpler and more accurate method described below.

General Description of Method. A graduated series of dilutions of each fluid is made in miniature test-tubes in practically the same manner as in the determination of the agglutinability. By artificial light against a dark background the turbidities of a number of tubes of one series are matched in succession with tubes of the other series. Each reading provides figures from which the relative turbidities can be calculated, and by taking the average of a number of readings an accurate ratio is obtained.

Details of Method. Apparatus. Almost any variety of dwarf test-tube or agglutination-tube may be used. It is essential to have at hand a fairly large supply of tubes, so that a selection may be made of tubes of practically equal internal diameter. The tubes must be quite clean and their surface must be free from scratches. We have been employing for the purpose two varieties of tubes which are made by Baird and Tatlock, Ltd. for the National Medical Research Committee, viz. (1) A thin-walled dwarf test-tube about 7·0 cm. long and 0·95 to 1·0 cm. in diameter. (2) A smaller "agglutination tube" 5·7–5·8 cm. in length, having an internal diameter of 5·5–6·2 mm. When the greatest possible accuracy is desired, it is best to use the larger form of tube and to measure the fluids with a 2·0 cc. pipette, accurately graduated in hundredths of a cc. But for ordinary purposes the small agglutination tubes may quite well be employed, and the dilutions made with a dropping pipette. In this latter method there is a certain error due to the difference in size of the drops of 0·85 % NaCl solution and of culture. About 17–18 drops of culture are equal to 15 drops of saline. But in dealing with small volumes of fluids, it is open to doubt whether measurements with a small graduated pipette are any more accurate than this.

Technique (when small agglutination-tubes and a dropping pipette are employed). Two sets of eleven gauged tubes are placed in a suitable stand. Into the tubes of each set the different fluids are measured as follows:

Tube	Saline	Culture
(1)	0	20
(2)	4	16
(3)	8	12
(4)	10	10
(5)	12	8
		Culture diluted 1 in 2
(6)	6	14
(7)	8	12
(8)	10	10
(9)	12	8
(10)	14	6
(11)	16	4

This series of dilutions is similar to that described for the serum-dilution in the standardisation of agglutinable cultures, only multiplied by two. It will be seen that the range of dilutions in the series is from 1/1 to 1/10, and that the progressively diminishing quantities of culture are always made up to the same volume with saline. The mixtures in the tubes are well shaken with the finger over the mouth of the tube, and the readings are then taken.

Readings. Artificial light and a black background are essential. It has been our custom to use a frosted electric bulb with a large green shade, and some pieces of dull black boarding or paper placed on the table below the lamp and propped up behind it. A darkened room, though not absolutely necessary, adds considerably to the ease and accuracy of the readings. The lamp shade is inclined so that the bulb is just hidden from the eye, and the tubes to be compared are held up against the edge of the shade. Before reading, the outside of the tubes must be wiped with the moistened corner of a glass-cloth and then polished with the dry part of the cloth. From one series a tube is chosen at random which we call the "standard" tube for the first reading. With this the tubes of the other series are compared one after the other until one is found which matches it in turbidity. It will frequently be found that there is no tube in the second series which gives an exact match, but that there is one tube which is a little more turbid, while the next lower is a little less. In this case the standard tube is recorded as lying between those two tubes of the other series.

Moreover it is usually possible to judge whether it is nearer to one than to the other. If so, we make a mark or marks under the number of the tube to which it is nearer. Five or six readings should be made in this manner, taking a number of tubes in succession as standard. It is well to read in both directions; *i.e.* to take tubes from each series as standard. Now, since we know the dilution of the culture in every tube, it needs only a simple calculation to arrive at the relative turbidity of the two cultures. Records of typical routine estimations are given in Tables I to IV. Experiment III was performed with small tubes and a dropping pipette; I and II were made with larger tubes and a 2·0 cc. pipette. When the latter procedure is followed there is no need to make a 1 in 2 dilution of the culture for the latter part of the series. Moreover twelve tubes may be used with advantage instead of eleven.

Thus: culture (cc.) 1·0, 0·8, 0·6, 0·5, 0·4, 0·35, 0·3, 0·25, 0·2, 0·17, 0·13, 0·10. Water (cc.) 0·0, 0·2, 0·4, 0·5, 0·6, 0·65, 0·7, 0·75, 0·8, 0·83, 0·87, 0·9. The range of dilution is the same as before; and the only difference is that the series is fuller by one tube at the right-hand end, which renders readings in this neighbourhood more accurate.

Moderate variations in the colour-intensity of the fluids do not appreciably affect the accuracy of the readings, for the progressive dilution renders such differences negligible at an early point in the series. If one of the fluids under comparison is so thick that the range of dilutions described proves insufficient to allow a comparison with the other fluid; then a further series of tubes must be set up in precisely the same manner, but using the thicker fluid diluted (1 in 10 or 1 in 20) instead of at full strength.

In practice it is often convenient to make a rough estimate of the relative thickness of the fluids before proceeding to the complete test. This is easily done by taking a small quantity of the two fluids in two tubes of equal calibre, and diluting the thicker fluid until the two show approximately equal turbidity. From this rough test it is easy to make an approximate calculation of the relative thickness of the fluids. If one fluid appears to be four or five times thicker than the other, it is best to dilute it suitably before putting up the test-series.

TABLE I.

Estimation of relative turbidities of fluids A and B.

A is a stock "standard" culture.
B is a fresh, undiluted, formalised bouillon culture of *B. Typhosus.*
Technique: graduated 2·0 cc. pipette and large tubes.

Readings			Actual quantities of culture in the matched tubes				
Series *A* Tube		Series *B* Tube	*A*		*B*	Ratio $\frac{\text{quantity of } A}{\text{quantity of } B}$	Deviation from mean in %
2	matches	7	0·80 cc.	=	0·30 cc.	2·67	0·37
3–$\underline{4}$	,,	9	0·53*	=	0·20	2·65	1·12
$\underline{4}$–5	,,	10	0·46	=	0·17	2·71	1·12
2–$\underline{3}$	,,	8	0·67	=	0·25	2·68	0·00
$\underline{6}$–7	,,	11	0·35	=	0·13	2·69	0·37
						Mean 2·68	Mean error 0·60 %

* This figure is obtained by interpolation. The reading 3–$\underline{4}$ signifies "between tubes 3 and 4, but rather nearer 4." Now tube 3 contains 0·60 cc. and tube 4 contains 0·50 cc.—so we may take the figure to be a little less than 0·55, viz. 0·53.

The average-figure given at the foot of the third column is an expression of the fact that 1·0 cc. of *B* gives the same turbidity-reading as 2·68 cc. of *A*, when both are diluted to the same volume. If we now wish to dilute *B* to the same turbidity as *A*, all we have to do is to add to each 1·0 cc. of *B* 1·68 cc. of diluting fluid.

The readings vary, as a rule, about 3 % to 5 % on either side of the mean, and it is uncommon to find a reading with an error so great as 10 %. Such a close correspondence of the individual readings, each of which is in itself an experiment, is sufficient evidence of the trustworthiness of the method.

TABLE II.

Estimation of relative turbidities of fluids A and B.

A is a stock standard culture.

B is a fresh, undiluted, formalised broth culture.

Technique: graduated 2·0 cc. pipette and large tubes.

Readings		Actual quantities of culture in the matched tubes			
Series *A* Tube	Series *B* Tube	*A*	*B*	Ratio $\frac{\text{quantity of } A}{\text{quantity of } B}$	Deviation from mean in %
5 matches	9	0·40 cc.	=0·20 cc.	2·00	0·50
2 „	5	0·80	=0·40	2·00	0·50
6 „	10	0·35	=0·17	2·06	2·49
3 „	7	0·60	=0·30	2·00	0·50
7 „	10–11	0·30	=0·15	2·00	0·50
				Mean 2·01	Mean error 0·90 %

Calculation for diluting *B* to the same turbidity as *A*:

$$1{\cdot}0 \text{ cc. of } B = 2{\cdot}01 \text{ cc. of } A.$$

Therefore, to each 1·0 cc. of *B* add 1·01 cc. of diluting fluid.

TABLE III.

Method: small Dreyer's agglutination tubes and dropping pipette.

A is a stock standard culture.

B is a fresh, undiluted formalised bouillon culture.

Readings		Actual quantities of culture in the matched tubes			
Series *A* Tube	Series *B* Tube	*A*	*B*	Ratio $\frac{\text{quantity of } A}{\text{quantity of } B}$	Deviation from mean in %
4 matches	9	0·500 cc.	=0·200 cc.	2·500	0·16
3 „	8–9	0·600	=0·245	2·450	2·16
2 „	6–7	0·800	=0·315	2·540	1·44
1 „	5–6	1·000	=0·395	2·530	1·04
6 „	10–11	0·350	=0·140	2·500	0·16
				Mean 2·504	Mean error 0·99 %

Although we have paid special attention to the use of this method in the preparation of standard agglutinable cultures, the method itself is not limited to any such special use. As an instance of its application in quantitative chemistry we give in Table IV an experiment in the estimation of minute quantities of silver chloride in suspension or colloidal solution.

The close correspondence between the values obtained by our turbidity-method and the values calculated from the known strengths of the solutions demonstrates that this simple technique is at least as accurate as the more elaborate methods of turbidity-estimation hitherto employed in the quantitative measurement of dilute inorganic solutions.

Description of Experiment shown in Table IV. The estimation of the relative strength of two dilute $AgNO_3$ solutions—by quantitative conversion into AgCl and turbidity-measurement. The $AgNO_3$ solutions were kindly prepared for us by Mr N. V. Sidgwick, and were labelled A = between N/100 and N/1000, and B = between N/1000 and N/10,000. Their true concentrations, which were unknown to us until after the experiment was completed, were:

$$A = 0{\cdot}001254 \text{ N } AgNO_3,$$
$$B = 0{\cdot}0004013 \text{ N } AgNO_3.$$

After a rough preliminary experiment, solution A was diluted one in four, to bring it down to a strength sufficiently near that of solution B. A thus diluted is referred to as A (0·25).

To 10 cc. of solution A (0·25) 10 cc. of 0·85 % NaCl solution was added.

To 10 cc. of solution B 10 cc. of 0·85 % NaCl solution was added.

The mixtures were kept for four hours in a cool and dark place, and then the resulting suspensions (or colloidal solutions) of AgCl were subjected to a turbidity-estimation in the usual manner. Both solutions showed a bluish opalescence without any visible trace of flocculation. Large tubes and a graduated 2·0 cc. pipette were employed for the measurements.

TABLE IV.

Turbidity-estimation of two silver chloride suspensions (or colloidal solutions).

A and B are two $AgNO_3$ solutions, converted quantitatively into AgCl (see text).

Readings A (0·25) B Tube	A Actual quantities of AgCl suspensions A (0·25)	B	Ratio observed $\frac{0{\cdot}25A}{B}$	Deviation from mean in %	Ratio calculated $\frac{0{\cdot}25A}{B}$
1=2–3	100	76	1·316	+2·41	
3=4–5	60	47	1·277	−0·62	$A = 0{\cdot}001254$ N $AgNO_3$
5=6–7	40	33	1·212	−5·68	$B = 0{\cdot}0004013$ N $AgNO_3$
7=8	30	25	1·200	−6·61	$\frac{0{\cdot}25A}{B} = \frac{{\cdot}0003135}{{\cdot}0004013} = \frac{1}{1{\cdot}280}$
7–8=9	27	20	1·350	+5·06	
5–6=7	38	30	1·267	−1·40	
3–4=5	52	40	1·300	+1·17	
2–3=4	68	50	1·360	+5·84	
			Mean 1·285	Mean error 3·60	1·280

Percentage divergence of observed from calculated ratio −0·39.

APPENDIX.

Gauging of the Tubes. For the selection of tubes of almost equal bore, a gauge is necessary. The following type has been found very convenient (Fig. 1). From a thin strip of brass a piece about an inch long is cut in the shape of a longitudinal section of a long truncated cone. The narrower end has the width of the minimum internal diameter allowable for the selected tubes (see below) and the wider end, to which a brass rod is soldered as a handle, has the width of the maximum internal diameter permissible. The gauging of the tubes consists in choosing those tubes whose mouths permit the entry of the narrower end of the gauge but refuse to admit the broader end. (A temporary but perfectly serviceable gauge of this kind can quickly and easily be cut from a small strip of thin wood to any designed measurement.) For the larger tubes the maximum and minimum measurements of the gauge may conveniently be made 9·3 mm. and 9·0 mm. respectively; and for the smaller tubes 5·7 mm. and 6·0 mm. respectively.

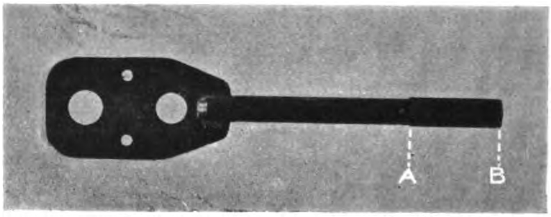

Fig. 1. Composite gauge for agglutination-tubes and dropping pipettes.

At one end of the instrument is seen the maximum (*A*) and minimum (*B*) gauge for the internal diameter of the agglutination-tubes. This is the only part that is described in the text. The holes in the plate at the other end are for the gauging of the external diameters of the tubes and of the capillary end of the dropping-pipettes. This part of the instrument is not essential for the method we have described.

Although this type of gauge measures only the mouth of the tube, and gives no information about its lower end, we have not found that this introduces any appreciable error, since the tubes are not long enough to show any considerable variation in width from end to end.

It is hardly necessary to say that the more uniform the bore of the selected tubes is, the greater will be the accuracy of the turbidity-readings.

SUMMARY.

A description has been given of a simple and accurate method of measuring the relative turbidity or opacity of two fluids; and its special application to the standardisation of agglutinable cultures has been described in detail.

The method is of general application in all chemical and biological investigations which require the measurement of turbidity.

The technique is extremely simple and no special or expensive apparatus is needed.

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XXXIV. ANAPHYLAXIS TO THE SEPARATED PROTEINS OF HORSE-SERUM.

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The earliest attempt to examine the part played by the different serum proteins in anaphylaxis to a foreign serum was made by Gay and Adler [1908]. Gay and Southard had based a theory of the anaphylactic condition on an erroneous interpretation of the phenomenon of passive anaphylaxis. This theory explained the sensitive condition as due to a remnant of the foreign serum, introduced at the sensitising injection, and still present in the system. Clearly it might be expected that the remainder thus conferring sensitiveness would belong to a different fraction of the serum protein from that which, at the second injection, acted as a poison. In accordance with this expectation, Gay and Adler found that the euglobulin fraction of a serum had great power of producing sensitiveness to the serum from which it was separated, but no toxic action when reinjected into the sensitised animal. Taking further fractions they observed a progressive decline of sensitising power, and increase of toxicity to the sensitised animal, as the proportion of ammonium sulphate needed for precipitation became greater; so that the highest fraction, the albumin, had practically no power of producing sensitiveness, but a maximum toxicity for animals sensitised with the whole serum, or with the lower fractions. These conclusions are in complete disaccord with the later results, obtained by various investigators, on anaphylaxis to pure proteins, of animal or vegetable origin. Such results have indicated a considerable degree of specificity of the reaction, not only for the species from which the protein originated, but even for pure proteins separated from an organ or tissue.

Doerr and Russ [1909, 1], repeating Gay and Adler's experiments with more attention to quantitative detail, obtained an entirely different result. Like Gay and Adler, Doerr and Russ worked with guinea-pigs, and separated the proteins of the ox or horse-serum used for sensitising by fractional precipitation with ammonium sulphate. They found, however, that sensitising power, and toxicity for the sensitised animal, declined in a parallel manner, with increase of the proportion of ammonium sulphate necessary for precipitation; so that the euglobulin fraction exhibited the maximum anaphylactic activity in either direction, the albumin being, in both respects, practically inert, and the pseudoglobulin occupying an intermediate position. Doerr and Russ's results are in conformity with the general tendency of the evidence obtained with other proteins. The only anomaly is the failure of the albumin fraction to act as an anaphylactic antigen. In a later paper [1909, 2], however, they showed a parallel failure of serum-albumin to produce a precipitin when injected into the rabbit, and, in conformity with their view of the identity of anaphylactic antibody with precipitin, a failure of serum from a rabbit so injected to confer passive anaphylaxis on the guinea-pig.

This question of the antigenic properties of the separate serum proteins, with regard to precipitin formation, has been examined by several other investigators, with results which show the widest possible variation, both in respect of the relative efficacy shown by the different proteins in exciting precipitin formation, and of the specificity to the individual proteins of the precipitins obtained. Some agree with Doerr and Russ as to the absence of a precipitin reaction to serum albumin; others, while obtaining a precipitin by injection of albumin, find that it precipitates even more strongly with globulin than with the albumin exciting its production. Doubtless a good deal of the variation is due to the varying degrees, in which the different methods employed effected a clean separation of the different proteins. The most convincing account is that given by Hunter [1905], whose paper may be consulted for the earlier literature. Hunter found that each of the serum proteins, euglobulin, pseudoglobulin, and albumin, was capable of exciting precipitin formation, and that the precipitin obtained in each case reacted most strongly with the protein used in its production, but in a modified degree with the others also; in other words, each precipitin showed a relative, or quantitative, but not an absolute, or qualitative specificity for its own protein.

Certain points in the behaviour of the serum-proteins as anaphylactic antigens seemed to deserve further investigation. Doerr and Russ's description of the progressive weakening of the anaphylactic effect in passing from the euglobulin to the albumin fraction, the pseudoglobulin being intermediate in activity, suggests a possibility that euglobulin may be the only protein in serum capable of producing the reaction, and that the weaker reaction of pseudoglobulin may be due to incomplete separation. The point is not without practical significance, as well as theoretical interest. The antibody of immune horse-serum has been shown to be associated with the pseudoglobulin fraction, and the various methods of artificial concentration have aimed at eliminating the albumin and the euglobulin; but the details of the processes in actual use would seem to ensure a more thorough exclusion of the former. It is clear, on the other hand, if the euglobulin is the anaphylactically active constituent, and the albumin indifferent in this respect, that the aim of any process for purifying antitoxin should be as thorough a removal of euglobulin as is possible in large-scale working.

METHODS.

We have confined our attention to horse-serum, specimens of the three proteins from which were available for use. Those which we used in the majority of the experiments were prepared by one of us for a chemical investigation, the results of which have already been published, together with the details of the methods of preparation [Hartley, 1914]. The euglobulin used was that prepared by Panum's method, and probably made a nearer approach to complete purity from the other proteins than did the specimens of pseudoglobulin and albumin, although these were probably as pure as repeated salting-out could make them. An additional specimen of the pseudoglobulin of horse-serum was kindly placed at our disposal by Dr H. Chick, who, after the usual separation by ammonium sulphate, had further purified it by continuous dialysing, with removal of the euglobulin thrown out of solution, during some months. Both the samples of pseudoglobulin were "euglobulin-free," in the sense that the ordinary methods of detection and separation would fail to discover euglobulin in them. But the quantity of a protein needed for sensitisation of the guinea-pig has been shown to be so minute, that it cannot be safely assumed that either was free from such traces of euglobulin as might be perceptibly effective in this direction. The same may be said with regard to a possible contamination of the albumin with minimal remnants of pseudoglobulin. In addition to the specimen above mentioned, a sample of albumin prepared in a different way was used in several experiments. This was a crystalline albumin prepared by Hopkins and Pinkus's [1898] method, and recrystallised three times, only that fraction being taken of each crop which consisted wholly of large well-formed crystals. This was dialysed free from ammonium sulphate and dried to a scale in vacuo. A portion of it was, for another purpose, dissolved in water, precipitated with cold acetone and ether, extracted for three days with warm ether, and finally taken up in water and recrystallised by Hopkins' method, yielding particularly well-formed crystals. These were again dialysed, scaled, and kept dry. Neither of these preparations showed any notable difference in action from the albumin-fraction obtained by salting-out in the ordinary way.

Most of our experiments were made on guinea-pigs, which were sensitised by hypodermic injection of a small dose, either of whole fresh serum, or of a solution of one of the separated protein preparations. After an interval, varying from 12 up to 31 days, the sensitiveness of the uterine plain muscle to two or more of the separated proteins was tested by the method previously described by one of us [Dale, 1912]. The use of this method has the definite advantage, over experiments made on a series of similarly prepared animals, that no allowance need be made for individual differences of sensitisation. The reaction of one horn of the uterus is tested with one of the pure proteins, and subsequently the reaction of the other horn, which can be regarded with some certainty as equivalent in specific sensitiveness and physiological condition to the first, is tested similarly with another of the pure proteins. Moreover the degree, to which an effective dose of either protein affects the subsequent reaction to the other, can be readily ascertained. By a careful choice of the order in which the doses are given, as much information can often be obtained from one such experiment, with regard to the relative sensitiveness to the different proteins, as would require the injection and reinjection of a long series of animals, if the more usual method of studying anaphylaxis in the guinea-pig were adopted. For purposes of control a few experiments were made by the ordinary method of intravenous reinjection into the intact animal, with observation of the degree of severity of the resulting symptoms as an index of the sensitisation. Young virgin female guinea-pigs were chosen, weighing about 180 g. at the time of the preparatory injection, so that the test of sensitisation, after an interval of two to three weeks, or more, was made when the animals weighed about 250 g. on an average.

The apparatus used was arranged, in all material particulars, similarly to that described by Dale and Laidlaw [1912]. The vessel in which the uterine horn under experiment was suspended contained, in all cases, 70 cc. of Ringer's solution. The "control" uterine horn was kept, till required, in a tube of the same solution, immersed in the same constant-temperature bath as the experimental vessel. The contents of the experimental vessel, and of the tube containing the control, were oxygenated and stirred by a stream of bubbles. The temperature employed was between 37° and 39° C. The lever recording the uterine contractions magnified twice or thrice, according to the size and activity of the uterus under observation. Similarly the weighting of the lever was varied so as to secure, in each case, efficient extension, without imposing undue resistance to contraction.

The Ringer's solution used was made up according to one or other of the formulae mentioned by one of us in an earlier paper. The results obtained were of the same kind with either, but we gathered the impression that, for keeping the general sensitiveness of the control horn at the same level as that first tested, during a long experiment lasting some hours, the solution called "Ringer I" in the earlier paper was, on the whole, the better. The various proteins tested were made up in Ringer's solution, usually in 1 % solution. The albumin and pseudoglobulin could be dissolved directly in the Ringer's solution without difficulty. Euglobulin so treated was imperfectly soluble. Although the imperfect, opalescent suspension, obtained with Ringer's solution alone, seemed to act perfectly well on the plain muscle when added to the bath, it was considered better, after the first few experiments, to use it in a more highly dispersed condition, in order to obtain a more accurate comparison of its effect with that of pseudoglobulin. With this object, the required amount was weighed out and rubbed up with a little Ringer's solution into a fine emulsion. To this was added 0·1 cc. N/10 NaOH for each 100 mgm. of euglobulin. The relatively clear solution so obtained was then diluted with Ringer's solution to a strength of 1 % euglobulin; the proportion of added soda, after such dilution, corresponding, therefore, to 1/1000 normal. Controls showed that a pure solution of soda, in this strength, when added to the bath in volume corresponding to those of the test doses of euglobulin solution, produced no noteworthy effect on the uterine activity.

A few experiments were also made on anaesthetised dogs, with record of the blood-pressure, to confirm the general applicability of some of the results obtained with guinea-pigs.

RESULTS.

Animals sensitised to whole horse-serum.

We first tested the reaction, to the pure serum proteins, of uterine muscle from guinea-pigs sensitised by a hypodermic injection of 0·01 or 0·1 cc. of whole horse-serum, given two to three weeks previously.

(*a*) *Euglobulin and Pseudoglobulin.* In two cases, out of some eighteen guinea-pigs tested, sensitisation, for some unexplained reason failed, so that the uterine muscle responded but weakly even to large doses of horse-serum. Except in these instances the plain muscle of guinea-pigs, which had received a sensitising injection of whole horse-serum, exhibited a pronounced sensitiveness to the euglobulin and pseudoglobulin of horse-serum. The sensitiveness to the two showed a general parallelism, so that, if one horn of the uterus gave a large contraction in response to 1 mgm. of euglobulin, the second horn usually reacted similarly to 1 mgm. of pseudoglobulin. Similarly, if the first horn tested gave only a small reaction with 1 mgm. of euglobulin, and a subsequent maximal reaction with 10 mgms. of the same, it was usually found that the second horn gave effects of the same order with the same doses of pseudoglobulin.

At first sight, therefore, the results might easily have been interpreted as signifying that the euglobulin and pseudoglobulin were acting as one common antigen. It will be shown later that the effects of sensitising by the separate proteins, instead of by whole serum, exclude such a supposition; but there were clear indications of the distinct antigenic properties of the two globulins, even in the experiments with whole serum. Such indications were obtained by studying the effects of desensitisation. In an earlier paper it was shown that the application of an adequate, effective dose of an antigen, to which the plain muscle has been made anaphylactic, will permanently remove its sensitiveness to that antigen, while leaving it, in other respects, practically normal. The question therefore arises, whether a dose of euglobulin, which renders indifferent to further euglobulin a preparation originally sensitive to both globulins, will at the same time remove its initial sensitiveness to pseudoglobulin, and *vice versâ*. There are three possibilities, and we have found examples of each, in our series of guinea-pigs sensitised by whole serum.

(i) The two globulins may appear to act as a common antigen, so that one horn may respond by a maximal contraction to a certain dose of euglobulin, and be thereafter insensitive not only to euglobulin, but to pseudoglobulin in like dosage; while the second horn may show an exactly parallel response to, and complete desensitisation by a like dose of pseudoglobulin. This type of reaction is illustrated by the following experimental record, Figs. 1 and 2 reproducing the corresponding tracings.

Guinea-pig No. 9. Sensitised by hypodermic injection of 0·1 cc. of whole horse-serum 16 days previously. Bath-volume 70 cc. Ringer's solution changed after the effect of each dose was complete.

1st horn of uterus (Fig. 1).

1 mgm. of euglobulin. Moderate contraction.
10 mgm. of euglobulin. Maximal contraction.
10 mgm. of pseudoglobulin. Nil.

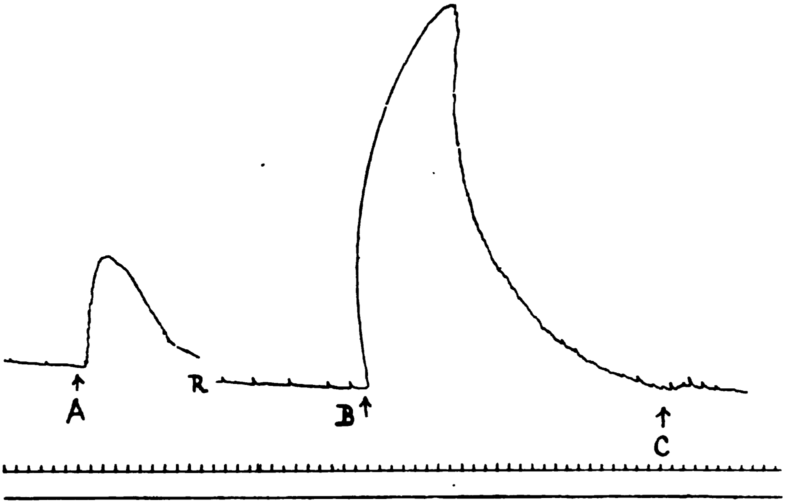

Fig. 1. Guinea-pig sensitised with 0·1 cc. of whole horse-serum. 16th day. 1st horn A. 1 mgm. of euglobulin. B. 10 mgm. of euglobulin. C. 10 mgm. of pseudoglobulin. Time marker (in all figures) marks intervals of 30 seconds. R (in all figures) indicates change of Ringer's solution.

2nd horn of uterus (Fig. 2).

1 mgm. of euglobulin. Moderate contraction.
10 mgm. of pseudoglobulin. Maximal contraction.
10 mgm. of euglobulin. Nil.

It will be seen that a dose of 10 mgm. (1 in 7000) of either euglobulin or pseudoglobulin produced a maximal response of the muscle, after the sub-maximal effect of 1 mgm. of euglobulin had been obtained; but that the maximal response, to 10 mgm. of either, left the organ insensitive to the same dose of the other. Such a result is not uncommon, and seems to be especially frequent in cases, such as that quoted, in which the sensitiveness is not of a high order. It may be noted that the apparently rather smaller amplitude of contraction in response to 10 mgm. of pseudoglobulin (Fig. 2) as compared with that to 10 mgm. of euglobulin (Fig. 1), is attributable to the use of Ringer II in the former, and Ringer I in the latter case, for the purpose of comparing their suitability for this type of experiment. It is no index, therefore, of preferential sensitisation, which is excluded by the perfect mutual desensitisation.

(ii) There may be sensitisation, to either euglobulin or pseudoglobulin in higher degree than to the other. The difference was in no case very conspicuous, and its demonstration depended chiefly on the phenomena of desensitisation. Thus we might find that one horn responded maximally to 1 mgm. of euglobulin, gave a second smaller response to 10 mgm. of euglobulin, and was thereafter desensitised not only to euglobulin, but to pseudoglobulin also; while the second horn after practically similar responses to 1 mgm. and 10 mgm. of pseudoglobulin, would be completely insensitive to this protein, but still capable of giving a large response to 10 mgm. of euglobulin. In another case the reverse condition would obtain, the plain muscle desensitised to pseudoglobulin failing to respond to euglobulin, while the second horn, when desensitised to euglobulin, still responded to pseudoglobulin. A couple of examples will suffice to illustrate the point, the importance of which was that it gave us the first indication of an antigenic distinction between the two proteins, which experiments on sensitisation with the separate proteins confirmed.

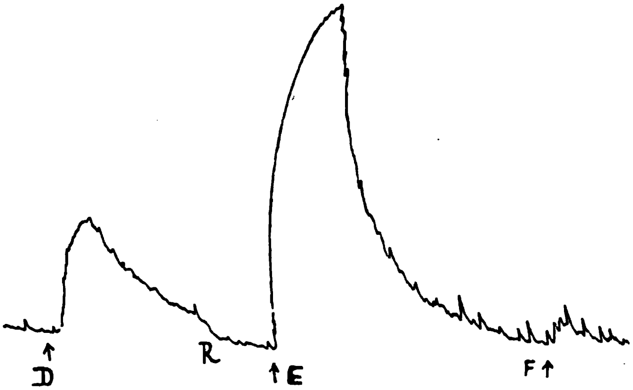

Fig. 2. From same experiment as Fig. 1. 2nd horn. D. 1 mgm. of euglobulin. E. 10 mgm. of pseudoglobulin. F. 10 mgm. of euglobulin.

Experiment 4. Guinea-pig sensitised with 0·1 cc. of horse-serum. 22nd day.

1*st horn.*

1 mgm. euglobulin.	Maximal contraction.
5 mgm. euglobulin.	Smaller contraction.
5 mgm. euglobulin.	Nil.
5 mgm. pseudoglobulin.	Nil.

2*nd horn.*

1 mgm. pseudoglobulin.	Maximal contraction.
5 mgm. pseudoglobulin.	Nil.
5 mgm. pseudoglobulin.	Nil.
5 mgm. euglobulin.	Large contraction.
5 mgm. euglobulin.	Nil.

Experiment 7. Guinea-pig sensitised with 0·1 cc. horse-serum. 21st day.

1*st horn.*

1 mgm. pseudoglobulin.	Good contraction.
1 mgm. pseudoglobulin.	Nil.
10 mgm. pseudoglobulin.	Small contraction.
10 mgm. pseudoglobulin.	Nil.
10 mgm. pseudoglobulin.	Nil.
10 mgm. euglobulin.	Maximal contraction.

2*nd horn.*

1 mgm. euglobulin.	Large contraction (? Maximal).
1 mgm. euglobulin.	Nil.
10 mgm. euglobulin.	Small contraction.
10 mgm. euglobulin.	Nil.
10 mgm. euglobulin.	Nil.
10 mgm. pseudoglobulin.	Nil.
40 mgm. pseudoglobulin.	Nil.

The above two records show preferential sensitisation to euglobulin. In the following the predominant sensitiveness to pseudoglobulin is shown not only in the greater effect of desensitisation to this protein on the subsequent response to euglobulin, but in the lower initial effective dose of the former.

Guinea-pig sensitised with 0·04 cc. antidiphtheritic horse-serum + 1 test dose diphtheria toxin. 24th day.

1st horn.

1 mgm. pseudoglobulin. Full contraction.
10 mgm. pseudoglobulin. Small contraction.
10 mgm. pseudoglobulin. Nil.
10 mgm. euglobulin. Slight contraction.

2nd horn.

1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Good contraction.
10 mgm. euglobulin. Moderate contraction.
10 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Very small contraction.
10 mgm. pseudoglobulin. Large contraction.
10 mgm. pseudoglobulin. Nil.

In this record there is an obvious indication that complete desensitisation to euglobulin is more difficult to achieve than that to pseudoglobulin. This point is clearly discernible in a number of our records. A probably related effect is also to be seen in the above, namely, the tendency of the plain muscle to recover from the desensitisation to euglobulin, and show a revival of response to this protein at a later stage of the experiment. This may indicate a tendency of the euglobulin-antibody complex to dissociate, or it may be attributable to the physical properties of the euglobulin solution, on account of which this antigen less readily reaches and saturates the antibody in the deeper layers of the uterine muscle. We have no direct evidence in favour of either interpretation, but the fact is repeatedly illustrated in our records.

(iii) In some of our records from guinea-pigs sensitised with whole horse-serum, the distinct antigenic properties of the two globulins are made evident by the fact that, while the plain muscle responds with equal readiness to an initial dose of either eu- or pseudoglobulin, desensitisation to either of these leaves a remainder of sensitiveness to the other. This is most readily shown by producing a *relative* desensitisation, by using small doses throughout, as in the following record.

Experiment 12. Guinea-pig sensitised with 0·1 cc. horse-serum. 14th day.

1st horn.

1 mgm. euglobulin. Good contraction.
1 mgm. euglobulin. Small contraction.

1 mgm. euglobulin. Nil.
1 mgm. pseudoglobulin. Fair contraction.
1 mgm. pseudoglobulin. Nil.

2nd horn.
1 mgm. pseudoglobulin. Good contraction.
1 mgm. pseudoglobulin. Nil.
1 mgm. euglobulin. Fair contraction.

It is probable, to judge from the results of other experiments, that the 1st horn, when no longer giving a perceptible response to 1 mgm. of euglobulin, would have responded to 10 mgm.; and similarly with pseudoglobulin in the case of the second horn. The point to be emphasised is that, in either case, the muscle, when reduced to a condition in which it fails to respond at all to 1 mgm. of one of the globulins, still shows a definite sensitiveness to the same dose of the other.

(*b*) *Albumin.* Our earlier results seemed to support the finding of Doerr and Russ, that serum albumin did not act as an anaphylactic antigen. In a series of preparations from guinea-pigs sensitised to whole serum, we found that the plain muscle seemed completely indifferent to a dose of 5 or 10 mgm. of the albumin, subsequently giving a maximal response to 1 mgm. of either globulin. The result seemed so definite, and was obtained with such regularity in the early series, that for some time we abandoned experiments with albumin, considering its inefficacy as settled. At a later stage of the investigation six guinea pigs received the usual sensitising injection of 0·1 cc. of horse-serum, to test another point, and it happened that the uterus was tested with 1 mgm. of pure serum albumin, which produced a typical, nearly maximal, response. The uteri of the other guinea-pigs of this series were then tested with albumin, and all showed a similar high degree of sensitiveness to this protein. The difference could not be attributed to a deficiently purified albumin, since the preparation which now proved an effective stimulus was the same as that which had previously proved ineffective; and the highly purified sample obtained by recrystallising four times, as described above, was quite as effective a stimulant as that purified by the precipitation method only. The only difference between the two series was in the time elapsing between the sensitising injection and the performance of the test. In the earlier series, in which no sensitiveness to albumin was detected, the interval was two to three weeks; in the later series, in which a good reaction to albumin was observed, it was upwards of thirty days—four to five weeks. A further series of six guinea-pigs was therefore prepared, being sensitised with 0·1 cc. horse-serum and kept for twenty-nine to thirty days before testing, with the same result, that after this interval a sensitiveness of the plain muscle to albumin had developed.

There was some indication that, concurrently with the late development of the sensitiveness to albumin, the sensitiveness of the plain muscle to the globulins, which attains its maximum somewhere between the 14th and 21st days, undergoes some decline, to judge by the size of the initial dose needed to produce a pronounced reaction. The following record illustrates the point.

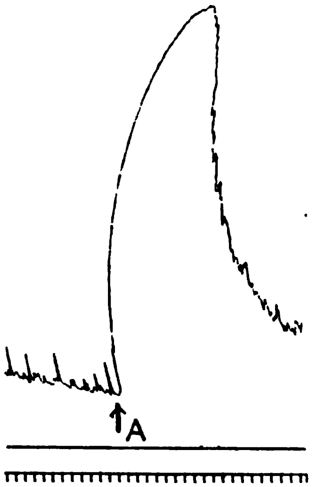

Fig. 3. Guinea-pig sensitised with 0·1 cc. of whole horse-serum. 31st day. 1st horn. A. 1 mgm. of albumin.

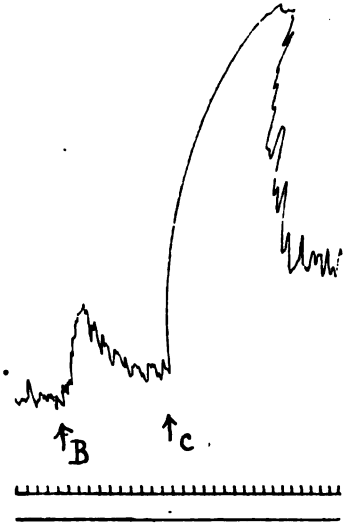

Fig. 4. Same experiment as Fig. 3. 2nd horn. B. 1 mgm. of euglobulin. C. 10 mgm. of euglobulin.

Experiment 26. Guinea-pig sensitised with 0·1 cc. horse-serum. 31st day.

1*st horn.*

1 mgm. albumin. Maximal contraction (see Fig. 3).
10 mgm. albumin. Weak contraction.
10 mgm. albumin. Nil.
1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Moderate contraction.

2nd horn.

1 mgm. euglobulin.	Small contraction.
10 mgm. euglobulin.	Maximal contraction (see Fig. 4).
10 mgm. euglobulin.	Nil.
10 mgm. albumin.	Nil.

It will be noticed that though the plain muscle, in this case, showed a higher sensitiveness to albumin than to euglobulin, as judged by its response to a small dose (1 mgm.) of either, it is more readily desensitised to albumin by an effective dose of euglobulin, than to euglobulin by albumin.

The results obtained, by observing the response of isolated plain muscle, suggested that the failure of previous workers to observe an anaphylactic reaction in the serum-sensitive guinea-pig, when pure albumin was used for the reinjection, might have been due to the allowance of an insufficient incubation-period for the development of sensitiveness to this protein. It seemed desirable, therefore, to confirm, by experiments on the intact animal, the fact that true anaphylaxis to serum albumin does occur, though late in its development; in other words, to ensure that the reaction of the isolated plain muscle, *in vitro*, gives, in this case also, a genuine indication of the occurrence of the anaphylactic condition, as seen in the whole animal. A guinea-pig from the same series as that used in Experiment 26, and similarly sensitised by a hypodermic injection of 0·1 cc. of horse-serum, was therefore tested on the 32nd day after sensitisation. The albumin was made up for the reinjection in 1 % solution in physiological saline. Of this solution 1 cc. (10 mgm. of albumin) was injected into the jugular vein, exposed by a small incision under local cocaine-anaesthesia. Symptoms appeared immediately after the completion of the injection, and typical anaphylactic death followed in about 3 minutes. The post-mortem appearances were quite characteristic—fixed distension of the lungs, and retarded coagulation of the blood.

It seemed desirable to confirm, in another species of animal, this efficacy of serum-albumin as an anaphylactic antigen, provided the incubation period is sufficiently long. A dog therefore received a sensitising injection of 5 cc. of horse-serum hypodermically. On the 28th day after this injection the dog was anaesthetised by A.C.E. mixture, after a preliminary injection of morphia. Arrangements were made for recording the blood-pressure from the carotid artery, and injection into the femoral vein. The record was started, and 20 cc. of a 5 % solution in saline of the pure albumin of horse-serum were then run slowly into the vein. A profound and typical shock occurred, the blood-pressure commencing to fall rapidly during the injection, continuing to fall after the injection was completed, and remaining long at a very low level. The fall of pressure was accompanied by the typical dyspnoea, and a sample of blood, obtained while the depression persisted from the other carotid artery, showed no sign of clotting after standing for 24 hours.

Guinea-pigs sensitised to individual proteins.

(*a*) *Sensitised to euglobulin.* Sensitisation with euglobulin produced a more clearly specific sensitiveness than that with either of the other proteins. When a guinea-pig was killed 13 to 17 days after a preliminary injection of 1 mgm. of euglobulin, its uterine plain muscle responded typically to 1 mgm. or 10 mgm. of euglobulin, but was apparently indifferent either to albumin or pseudoglobulin, at any rate within the limits of dosage employed in these experiments (see Figs. 5 and 6).

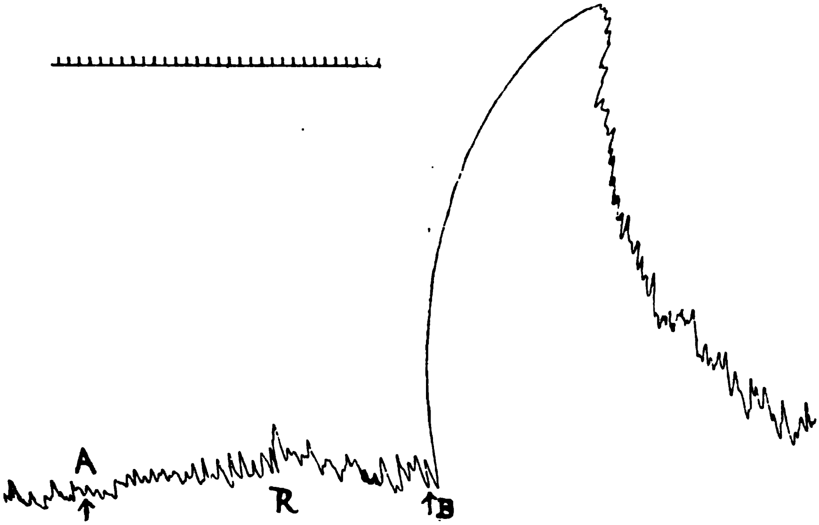

Fig. 5. Guinea-pig sensitised with 1 mgm. of euglobulin. 17th day. A. 5 mgm. of albumin. B. 1 mgm. of euglobulin.

(*b*) *Sensitised to pseudoglobulin.* The available samples of pseudoglobulin produced a sensitisation to either pseudoglobulin or euglobulin, but the sensitiveness to the former was always definitely superior, as the following record shows.

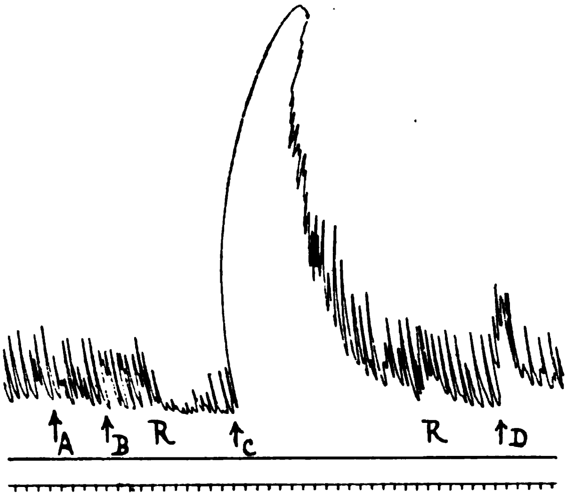

Fig. 6. Guinea-pig sensitised with 1 mgm. of euglobulin. 13th day. A. 1 mgm. of pseudoglobulin. B. 10 mgm. of pseudoglobulin. C. 10 mgm. of euglobulin. D. 10 mgm. of euglobulin.

Experiment 30. (See Figs. 7, 8 and 9.) Guinea-pig sensitised with 1 mgm. of pseudoglobulin. 22nd day.

1*st horn.*

1 mgm. pseudoglobulin. Maximal contraction.

2*nd horn.*

1 mgm. euglobulin.	Moderate contraction.
10 mgm. euglobulin.	Good but submaximal contraction.
10 mgm. euglobulin.	Small contraction.
10 mgm. euglobulin.	Nil.
10 mgm. pseudoglobulin.	Good but submaximal contraction.

It is clear that 1 mgm. pseudoglobulin, as a first dose, is much more effective than 1 mgm. euglobulin under the same conditions; further that complete desensitisation to euglobulin weakens, but by no means annuls the sensitiveness to pseudoglobulin.

Experiment 35. Guinea-pig sensitised with 0·1 mgm. pseudoglobulin 18 days.

1*st horn.*

0·1 mgm. pseudoglobulin.	Small contraction.
1 mgm. pseudoglobulin.	Moderate contraction.
1 mgm. pseudoglobulin.	Nil.
10 mgm. euglobulin.	Very small contraction.

2nd horn.

1 mgm. euglobulin.	Nil.
10 mgm. euglobulin.	Maximal contraction.
10 mgm. euglobulin.	Nil.
1 mgm. pseudoglobulin.	Small contraction.

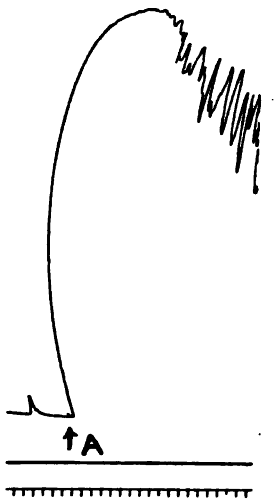

Fig. 7. Guinea-pig sensitised with 1 mgm. of pseudoglobulin. 22nd day. 1st horn. A. 1 mgm. of pseudoglobulin.

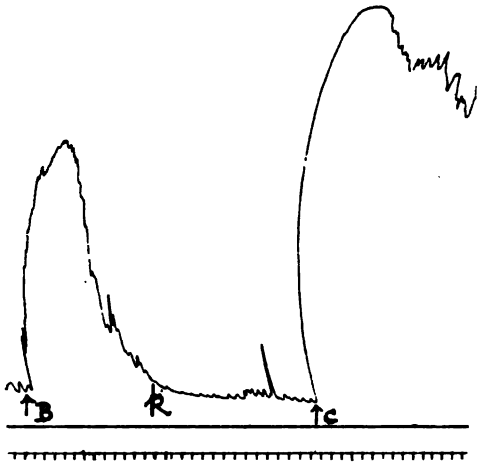

Fig. 8. Same experiment as Fig. 7. 2nd horn. B. 1 mgm. of euglobulin. C. 10 mgm. of euglobulin.

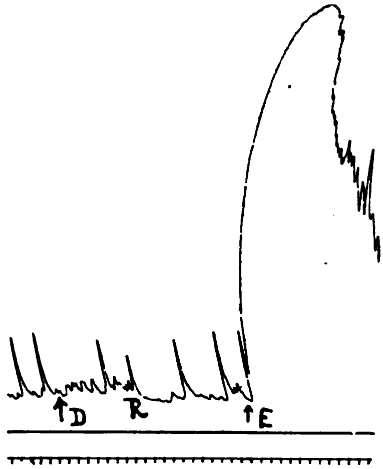

Fig. 9. Continuation of Fig. 8. D. 10 mgm. of euglobulin. E. 10 mgm. of pseudoglobulin.

In this experiment an error was made in dilution, which resulted in the dose of pseudoglobulin being throughout only one-tenth of what had been intended. The mistake, however, was not without advantage, in bringing out very clearly the preponderance of sensitiveness to pseudoglobulin. For the result showed that desensitisation to 1 mgm. of pseudoglobulin almost abolished the response to 10 mgm. of euglobulin; while after desensitisation to 10 mgm. of euglobulin, the muscle responded definitely, though not powerfully, to 1 mgm. of pseudoglobulin. It may also be noted that, whereas the first horn gave a small, but quite definite response even to 0·1 mgm. pseudoglobulin—*i.e.* to a dilution in the testing-bath of 1 in 700,000—the second horn gave no response at all to 1 mgm. (1 in 70,000) of euglobulin, but was stimulated to maximal contraction by 10 mgm. (1 in 7,000).

Sensitised to serum albumin.

All the guinea-pigs used in these tests received a sensitising hypodermic injection of 1 mgm. of purified serum albumin. Unfortunately lack of time, and the exhaustion of our small stock of purified pseudoglobulin, prevented any discriminating tests between the antigenic properties of pseudoglobulin and albumin, which would have given information of some interest. We were able, however, to show, more definitely than with the guinea-pigs sensitised to whole serum, the antigenic independence of albumin and euglobulin. Few experiments were made with isolated muscle, and it will be sufficient to quote one. It will be noted, both in this case and in the records of experiments on the intact animal, that when pure albumin is used for sensitisation, anaphylaxis to this protein, though still somewhat slowly developed, appears at an earlier stage than when the immunity reaction is complicated by the presence of the other serum proteins in the sensitising inoculum.

Experiment 35. Guinea-pig sensitised with 1 mgm. serum albumin hypodermically. 22nd day.

1st horn.

1 mgm. serum albumin. Good, but not maximal contraction.
10 mgm. serum albumin. Nil.
10 mgm. euglobulin. Nil.

2nd horn.

1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Trace of response (doubtfully specific).
1 mgm. albumin. Good, but not maximal contraction.

For the experiments on anaphylaxis *in vivo* two guinea-pigs (*A* and *B*) were first sensitised, each with 1 mgm. albumin as usual. Twenty-two days later, when each weighed 250 g. *A* was tested by intravenous injection of 10 mgm. albumin in 1 cc. of saline. This caused pronounced symptoms—coughing, chattering, erection of hair, falling on the side. After about 20 minutes, however, the animal was obviously recovering, and 10 minutes later was practically normal again. Next day it was perfectly well.

On the 35th day both guinea-pigs weighed about 275 g. *B* received 5 mgm. of albumin in 0·5 cc. of saline intravenously, showed immediate pronounced symptoms, and died within 2 minutes.

A, which had survived the injection on the 22nd day, was given a second injection of 10 mgm. on the 35th day. Symptoms developed slightly more slowly than in *B*, but death occurred within 3 minutes.

Both animals showed the typical post-mortem picture of acute anaphylaxis—lungs fixed in distension, slowly clotting blood, etc.

A further series of twelve guinea-pigs (numbered I–XII) were sensitised with 1 mgm. of albumin and examined as follows.

No. of guinea-pig	Weight in g.	No. of days after sensitisation	Dose given intravenously	Result
I	275	10	Albumin 20 mgm.	Slight symptoms, recovered
II	275	10	„ 10 „	Nil
III	275	17	„ 20 „	Death in 3 mins.
IV	250	17	„ 10 „	„ 4 „
V	250	17	„ 5 „	„ 5 „
VI	225	17	„ 2 „	„ 3 „
VII	215	17	„ 1 „	„ 3 „
X	220	27	Euglobulin 5 mgm.	Nil
XII	210	27	Albumin 0·5 mgm.	Death in 2 mins.
X (2nd injection)	220	28	„ 1 mgm.	„ 4 „
I (2nd injection)	300	30	Albumin 10 mgm.	Nil
II (2nd injection)	300	30	„ 1 „	Nil

Guinea-pig VIII was used for the isolated muscle test quoted above; guinea-pigs IX and XI died from accidental causes before testing.

There are several points worth noting in the above table. The sensitiveness to albumin is practically non-existent on the 10th day, by which sensitiveness to whole serum is well developed in animals previously injected with this. By the 17th day, however, in this series a very high sensitiveness to albumin has appeared, the lower limit of fatal dosage for guinea-pigs of this size not being reached in the experiment. The experiments on the 27th and 28th days show that there is no reaction to euglobulin in these guinea-pigs, even in a dose at least ten times as large as the fatal dose of albumin; nor does the injection of euglobulin notably modify the effect of albumin given on the next day. Lastly, it is seen that, when large injections have been given on the 10th day, *i.e.* before sensitiveness to albumin has appeared, no sensitiveness of the animal to albumin can be detected by intravenous injections given on the 30th day. It has been shown by one of us that such lack of apparent sensitiveness may be due either to genuine desensitisation of the tissues, or to an excess of antibody in the circulating fluids, protecting from the antigen tissues which, when isolated, are highly sensitive. It must remain an open question, for the present, which of these two conditions accounted for the lack of intoxication in this instance.

NOTE ON ANAPHYLAXIS TO PURE EGG-ALBUMINS.

The production in the guinea-pig of a high degree of anaphylaxis to the pure, crystallised albumin of the hen's egg has been previously described by Armit [1910] and by Wells [1908]. Wells [1911] has also shown that ovovitellin, from egg-yolk, acted as a quite separate antigen from the proteins of egg-white[1]. In the preceding sections we have given evidence of the separate action, as anaphylactic antigens, of the different proteins of one serum. By way of contrast it seems appropriate to make preliminary mention here of some experiments made by one of us (H. H. D.) on anaphylaxis to the pure crystalline albumins from the egg-white of hens and ducks, in the preparation of which Dr A. J. Ewins kindly co-operated. The albumins were crystallised by the familiar method of Hopkins [1899], three successive crystallisations being given to each sample. The eggs were obtained from a private source, and there can be no question of any mistake in specific identification. The albumin from the ducks' eggs was rather more difficult to crystallise than that from hens, a larger addition of acid being required to initiate the first crystallisation. When once obtained in clean crystals, however, it was recrystallised as readily as that of the hen. The final crop of crystals, in both cases, was dialysed free from ammonium sulphate, and the watery solutions were evaporated to dry scale-preparations at room-temperature *in vacuo*, solutions being then prepared as required. Female guinea-pigs received injections of 0·1–1 mgm. of either albumin, and the isolated uterine plain muscle was tested for sensitiveness after three weeks. The result can be stated in a line. There was no evidence of any antigenic distinction between the two albumins. The plain muscle of guinea-pigs, which had been injected with hen's albumin was quite as sensitive to duck's albumin as to hen's, and the same was true of preparations from guinea-pigs injected with duck's albumin. Moreover an effective dose of either albumin was followed by complete insensitiveness to the other, irrespective of which had been used as the sensitising dose. If the notebook record had been lost, it would have been impossible, from the response of the plain muscle, to form any opinion as to which albumin had been used for sensitisation. Fig. 10 illustrates the corresponding reactions of the two uterine horns from a guinea-pig sensitised with hen's albumin. It may be added that neither preparation had any effect on uterine muscle from normal, unsensitised guinea-pigs.

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[1] There is a somewhat extensive literature on the crossed specificity of different egg-whites, which has not been dealt with here, since the experiments made by us at present have been concerned solely with the crystalline albumins.

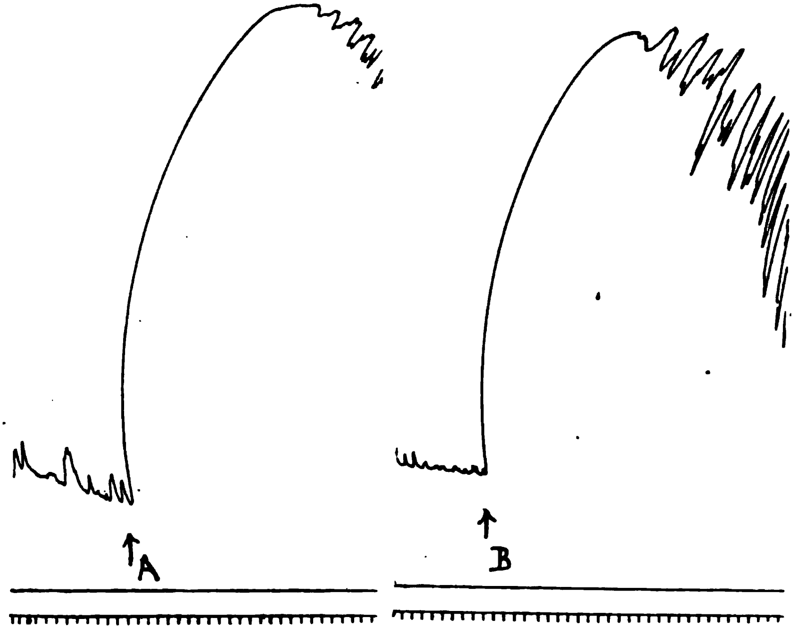

Fig. 10. Guinea-pig sensitised with 1 mgm. of crystalline albumin of hen's egg-white. 22nd day. A. 1st horn. Effect of 0·05 mgm. duck's egg-albumin. B. 2nd horn. Effect of 0·05 mgm. hen's egg-albumin.

SUMMARY AND DISCUSSION.

The following are the main conclusions to be drawn from the above experiments:

1. Each of the three proteins separable from horse-serum by their physical and chemical properties—euglobulin, pseudoglobulin, albumin—can act as an anaphylactic antigen.

2. A guinea-pig which has received a sensitising injection of one of these proteins is more sensitive to that one than to either of the others from the same serum. In some cases the sensitisation seems to be rigidly "specific," as when euglobulin produces sensitiveness to euglobulin but not to pseudoglobulin, or when albumin produces a high degree of sensitiveness to albumin but none to euglobulin.

3. The sensitiveness of the guinea-pig to albumin is later in development than that to the globulins; this difference possibly accounts for the fact that previous authors failed to detect the sensitising property of albumin. The difference is especially marked when sensitisation is carried out with whole serum, containing all three.

4. An effective dose of any of the proteins, to which the guinea-pig's plain muscle has been sensitised, partially or completely desensitises it to the other proteins of the same serum.

5. The crystalline albumins from the white of the eggs of hens and ducks show no distinction of antigenic properties.

The effects of "crossed" desensitisation, in particular, may give the impression that there is a considerable community of antigenic function among the proteins of one serum. Such an impression, however, is not completely justified, since one of us (H. H. D.) has previously shown that, when a guinea-pig is simultaneously sensitised to two or more antigens of widely different origin, and of undoubted antigenic independence—*e.g.* egg-white and horse-serum—desensitisation of the plain muscle to one may greatly weaken the sensitiveness to the other. On the other hand, the fact that sensitisation with one of the globulins frequently produces a smaller, but quite definite sensitiveness to the other, shows that, at any rate, the preparations of these two substances at our disposal were not completely independent in their antigenic properties. But the question necessarily arises whether this overlap is a genuine phenomenon, or is due to the fact that, even in such elaborately purified preparations as those which we used, the separation was not ideally perfect. This possibility is worthy of consideration, in that the amount of a protein needed to evoke some degree of sensitiveness is almost inconceivably small. Wells [1908], for example, succeeded in sensitising guinea-pigs with 1/20,000 milligram of egg-albumin. This explanation of the overlap in sensitisation is favoured by the evidence that it appears most prominently when the impurity of the sensitising protein is most likely to be considerable. The purification of euglobulin is facilitated by its insolubility in weakly acidulated water. We have found that a rigidly specific sensitiveness to euglobulin occurs comparatively frequently. On the other hand, we never succeeded, by injection of pseudoglobulin in producing a sensitiveness to pseudoglobulin alone; sensitiveness to euglobulin in addition was always detectable, though it might be relatively weak, especially when the sensitising dose was small. But the purification of pseudoglobulin from the last traces of euglobulin is so difficult, that it may be doubted whether it is possible by existing methods. It is similarly difficult to make a sharp separation between pseudoglobulin and albumin, and we think it probable that a similar overlap of sensitisation would have been detected between these two proteins. Unfortunately the material was exhausted before this point had been put to the test. Again, if we regard the overlap of antigenic properties as due to incomplete separation, it would be expected that the sharpest distinction would be observed between euglobulin and albumin, each of which can be obtained with tolerable certainty uncontaminated by the other. The results were quite in accordance with this expectation, since the plain muscle from a guinea-pig sensitised with euglobulin seemed quite indifferent to albumin, and *vice versâ*. On the whole, then, the evidence seems to us in favour of the view that the different proteins of a serum, in so far as they can be obtained pure from one another, act as independent anaphylactic antigens.

This narrowing down of immunological "specificity," to a discrimination between the different pure proteins of a single tissue of one species, is by no means new. Wells [1911], as above mentioned, found evidence of complete antigenic independence between the "ovovitellin" of egg-yolk, on the one hand, and the proteins of egg-white on the other; he found, however, a considerable overlap in the antigenic properties of the albumin and globulin of egg-white, however carefully they were purified. Ovomucoid was again an independent antigen. Wells and Osborne [1911, 1913] have furnished numerous instances in which the different pure proteins from one plant species act as separate antigens, while the corresponding pure proteins from allied species have common antigenic properties. Such evidence gives to the fundamental problem of immunity a more hopeful aspect, by its suggestion that the antigenic distinction is possibly dependent on such properties of the different proteins as may be open to the ordinary methods of chemical and physical enquiry, and not necessarily on some biological difference of so mysterious a nature, as to elude investigation by such methods. Our own evidence of the antigenic independence of the proteins from one blood-serum, and of the community of antigenic function between the pure albumins of egg-white from species so widely separated as the fowl and the duck, has the same tendency. It seems clear to us that every sensitisation with a whole serum, for example, is in reality a complex multisensitisation; and that it is essential to work with individual pure proteins, in any attempt to investigate the nature of the difference, of which antigenic independence is an expression.

The question has often been debated whether the difference is a chemical or a physical one, without giving these terms any greater precision of application. One possibility of chemical difference we are in a position to eliminate. An analysis of the separated proteins from horse-serum, used by us in these experiments, had been carried out by one of us [P. Hartley, 1914] by the aid of van Slyke's method. It was found that albumin, as others had shown, differed from the globulins in yielding, on hydrolysis, a higher proportion of the diamino-acids, and in containing a higher proportion of cystine. It was further shown that, of the diamino-acids, the excess yielded by albumin, as compared with the globulins, was greatest in the case of lysine, which albumin contained in about a 50 % higher proportion. No such difference was found between the two globulins, which, as regards the distribution of their nitrogen among the different amino-acids, showed no difference of any note. There is no help to be obtained, therefore, in this direction towards an explanation of the antigenic difference between the globulins. It is clear, however, that there is abundant room for difference of chemical structure even between protein molecules built up from the same amino-acids in identical proportions. Indeed, if the highly specific combining properties, which give a protein molecule its antigenic character, are related to chemical structure at all, it seems likely that they would be as much dependent on the pattern of the structure of the molecule, on the order in which the amino-acids are linked up in it, as on their nature and the proportions in which they are present. A relation between the stereo-chemical and antigenic characters of the protein molecule is, indeed, definitely suggested by the observation of Ten Broek [1914], who found that a protein racemised by Kossel's method had lost all power of acting as an antigen, just as Dakin and Dudley [1913] found that it had lost its susceptibility to the action of proteolytic enzymes. Dakin [1912] has given reason for believing that the amino-acids which escape racemisation in this process, and are therefore obtained in the natural, optically active condition on subsequent acid hydrolysis of the racemised protein, are those occupying the terminal positions of the peptide chains out of which the protein molecule is built. Dudley and Woodman [1915], using Dakin's method, found evidence of a structural difference of the kind under discussion between the caseinogens of cow's and of sheep's milk—proteins consisting of identical amino-acids in identical proportions. In the case of sheep's caseinogen the tyrosine wholly and the lysine largely escaped racemisation, while they were both completely racemised in that of cow's caseinogen. The instance is an unsatisfactory one for our present purpose, since the antigenic properties of caseinogen are relatively feeble, and the caseinogens from different species show no clear disparity of antigenic properties. In the only other case of proteins with identical amino-acid content which have hitherto been thus compared, the euglobulin and pseudoglobulin of colostrum, there is presumptive evidence of an antigenic difference of the same kind as that which we find existing between the corresponding serum-proteins. In this instance Dudley and Woodman detected no difference by the racemisation method. So that, so far as evidence of this kind has been obtained at present, it fails to support, and might even seem to discredit the view that antigenic properties depend on the structural pattern of the protein molecule. We have an example of structural difference, without antigenic disparity, and another, with no structural difference detected, in which some degree of antigenic independence may be presumed. It must be remembered, however, that the method reveals only one kind of structural difference, the effect of which on the antigenic properties might well be subsidiary to other differences, which the method fails to detect. The observation that two similar proteins from different species, the caseinogens of the cow and sheep, while as yet indistinguishable by other methods, show a definite difference in the order of amino-acid linkage, remains highly significant, and seems to indicate the lines along which the possibility of a connexion between antigenic properties and chemical structure may yet be investigated.

It has not been assumed, in thus considering the possibility of a chemical basis for antigenic specificity, that the combination between antigen and antibody is of the type of an ordinary chemical union. There is, of course, much evidence indicating that the association has the character rather of an adsorption. This, however, by no means precludes the possibility that it may be conditioned ultimately by the structure of the antigenic molecule. There is similarly strong evidence in favour of regarding the association between an enzyme and its substrate as an adsorption; yet it is essentially dependent on the configuration of the substrate molecule. Again, Barger and his co-workers [1915] have demonstrated the connexion between the formation of coloured adsorption-compounds with iodine and a certain type of structure in organic compounds. They also showed, however, the dependence of the reaction on surface-phenomena, in that a colloidal solution, or suspension of the substance in amorphous aggregates, was necessary for the formation of the typical coloured complex with iodine. It is not improbable that the antigenic behaviour of a protein is similarly conditioned by its state of dispersion. Miss Chick [1914] and others have given reason for considering euglobulin as possibly formed by association of pseudoglobulin with a lipoid, which gives to euglobulin its phosphorus content and its characteristic physical properties. It is conceivable that the antigenic difference between a pseudoglobulin and the euglobulin from the same serum may be due to the peculiar physical characteristics conferred on the euglobulin by associated lipoid. We hope later to test this possibility by comparing the antigenic action of a pseudoglobulin, with that of an artificial "euglobulin" made from it. But even if that were the case, such characteristics could hardly be more than one factor in the phenomenon of specificity; and to form an idea of its significance, it would be necessary to examine the degree of antigenic independence exhibited by pure euglobulins from the sera of different species. Using the purest proteins obtainable as antigenic units, it should eventually be possible to compare different proteins from the same species, and similar proteins from different species, in respect of their molecular configuration and also of their physical properties, and to trace the relation of each type of character to the antigenic individuality of the substances examined.

A word may be added on some practical bearings of our results. Since all the proteins of a serum can act as anaphylactic antigens, it is evident that the ideal to be aimed at, in concentrating the curative element in a specific immune serum, is simply the reduction of the ratio of total protein to antitoxic value; for the purpose of reducing serum-reactions, the elimination of albumin seems to be as important as that of euglobulin, when the pseudoglobulin is the fraction carrying the therapeutic power. Our observation of the relatively long latent period of the sensitiveness to albumin is also suggestive in connexion with the successive crops of serum rash, which have been recorded in certain patients as the result of one injection of a serum. It has been suggested [Goodall, 1907] that these represent separate reactions to the sera of the different horses which may have contributed to the serum. It seems to us more reasonable to suppose that they represent the successive appearances, at different time intervals, of sensitiveness to the different serum-proteins.

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XXXV. A COMPARATIVE STUDY OF THE PROTEINS OF THE COLOSTRUM AND MILK OF THE COW AND THEIR RELATIONS TO SERUM PROTEINS.

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Very diverse views have been propounded from time to time as to the nature of the protein constituents of cow's milk. On the one hand Duclaux and others have asserted that only one protein, caseinogen, is present, existing in coagulable and non-coagulable forms. On the other hand the literature of milk proteins includes claims to the isolation of some nine or ten distinct proteins. There can be little doubt that the majority of these latter preparations were of very doubtful purity, and from a close survey of the literature it is clear that the presence of only two distinct proteins has been demonstrated with reasonable certainty, viz. caseinogen and lactalbumin. In normal cow's milk of average quality these are present to the extent of about 3·0 % and 0·4 % respectively. A third protein, with the general characteristics of a globulin, was isolated by Sebelien [1885] and independently by Emmerling [1888], the amount present in milk being stated to be not more than about 0·005 %. More recently Halliburton [1890] has denied the existence of this lactoglobulin, the material isolated by Sebelien being regarded by him as lactalbumin.

There can be no such doubt as to the existence of a globulin in the colostrum—the fluid secreted in the earliest stages after parturition. The secretion at this stage is characterised by an extraordinary richness in protein material, this forming quite commonly 18 to 20 % of the total secretion. The proportions of caseinogen (4–5 %) and of albumin (0·7–1·5 %) are appreciably higher than in the normal milk of later stages of lactation, but the great bulk (6–12 %) of the colostrum protein consists of a globulin. With the development of mammary activity this preponderance of globulin rapidly vanishes, and usually within 48 hours of calving the proportion of globulin in the secretion has become similar to, or less than that of albumin, the total protein being correspondingly reduced.

These changes with advance of lactation in the proportions of the three proteins present are well illustrated by the following averages obtained by us with seven cows of the herd at Manor Farm, Garforth (Experimental Farm of the University of Leeds and the Yorkshire Council for Agricultural Education). The cows were milked twice daily after calving.

	Nitrogen present				
	Total %	as caseinogen %	as albumin %	as globulin %	as non-protein %
1st milking	2·40	0·75	0·14	1·32	0·19
2nd „	2·01	0·68	0·17	1·02	0·14
3rd „	1·44	0·59	0·14	0·59	0·12
4th „	0·97	0·51	0·11	0·31	0·04
5th „	0·76	0·46	0·07	0·20	0·03
6th „	0·75	0·46	0·06	0·20	0·03
7th „	0·69	0·42	0·06	0·18	0·03
8th „	0·65	0·46	0·05	0·12	0·02

A more prolonged series of analyses made by Sutherst [1902] with a single cow gave very similar results.

It has been commonly assumed that the protein preparations obtained from colostrum are identical with the similarly named preparations obtainable by the same methods from milk, but little evidence has yet been adduced in justification of this assumption. Even in the case of caseinogen the existing evidence amounts to little more than the close agreement in physical properties and ultimate chemical composition.

In the work described in the present communication we have endeavoured to obtain really conclusive evidence on this point by a study of the chief products of hydrolysis of preparations of caseinogen, globulin and albumin isolated with the greatest care from the colostrum and later from the normal milk of the same cows. Two cows (2AI and 10AI) of the dairy herd at the Manor Farm, Garforth, were used for the purpose, the colostrum being taken in each case immediately after calving, whilst three months later a quantity of the milk of each cow was worked up in precisely similar fashion.

The methods adopted for the isolation and purification of the different proteins are described fully later. For the characterisation of the proteins the method of analysis elaborated by van Slyke [1911] was used. This method has now become widely known and it will suffice here to indicate that it consists of a quantitative study of the distribution of the nitrogen of the protein between the different nitrogenous ingredients of the mixture obtained by acid hydrolysis of the protein. The determinations made lead finally to a classification of the nitrogen distribution under the following heads:

Ammonia-N, melanin- (or humin-) N, cystine-N, arginine-N, histidine-N, lysine-N, and nitrogen in forms not precipitable by phosphotungstic acid (subdivided into mono-amino-N and non-amino-N).

The method requires only 3-4 g. of protein, and with due allowance for experimental errors, all the nitrogen can be accounted for. The limits of accuracy which must be kept in mind in discussing the results may be gauged from the following data given by van Slyke [1911] which represent as large differences between duplicates as are normally to be expected.

	%		%
Ammonia-N	0·37	Lysine-N	1·23
Melanin-N	0·39	Total N of filtrate ...	1·50
Cystine-N	0·11	Amino-N of filtrate ...	1·10
Arginine-N	1·27	Non-amino-N of filtrate	1·20
Histidine-N	0·93		

It should be remarked that van Slyke's analyses, from which the foregoing table of errors is deduced, were done on duplicate portions of the same hydrolysis solution. In the experiments described in this paper, however, each protein was hydrolysed separately, and thus any errors incurred during the preparation of the proteins or during their hydrolysis would be added to the experimental errors described above.

In order to ensure the strictest possible comparability of the results throughout, the whole of the experimental work has been carried out by one of us (H. R.) and every effort has been made to secure the maximum of accuracy of which the method is capable.

The determination of the histidine-N, lysine-N and non-amino-N by difference rather than by direct estimation would seem to make these figures liable to considerable errors, but experience, as indicated above, shows that the errors are, in actual fact, relatively small [cf. van Slyke, 1911].

In addition to furnishing evidence as to the identity or otherwise of the corresponding proteins of colostrum and milk, our results, taken in conjunction with those obtained by the same methods by Hartley [1914] with the globulins and albumin of ox-serum, enable conclusions to be drawn as to the identity or otherwise of the globulins and albumin of colostrum and milk with the analogous proteins of ox-serum.

Further, since we have obtained lactoglobulin in pseudoglobulin and eu-globulin forms we have been able to compare the chemical structure of the protein in these two forms.

CASEINOGEN.

Evidence of the identity of caseinogen prepared from colostrum with that prepared from milk was first adduced by Sebelien [1885] and later by Winterstein and Strickler [1906]. In each case the evidence amounted to little more than the close agreement in ultimate chemical composition, and the similarity of response to familiar qualitative protein tests. Their conclusions have been substantiated, however, by the more recent work of Bauer and Engel [1911] and others in which the precipitin and other biological tests have been used.

Our own results are summarised in Table I, which gives the results of analyses of two preparations each from colostrum and milk.

TABLE I.

Summary of the Corrected Analyses of Different Preparations of Caseinogen in Percentages of the Total Nitrogen.

	From colostrum		From milk		Average		Average of all four samples
	(1) Cow 2AI %	(2) Cow 10AI %	(3) Cow 2AI %	(4) Cow 10AI %	Colostrum (1 and 2) %	Milk (3 and 4) %	%
Ammonia-N	10·42	10·07	10·15	10·35	10·25	10·25	10·25
Melanin-N	1·29	1·35	1·27	1·12	1·32	1·20	1·26
Total N of bases	25·94	26·76	27·52	26·28	26·35	26·90	26·62
Cystine-N	1·34	1·40	1·23	1·24	1·37	1·24	1·30
Arginine-N	8·95	9·87	9·73	8·71	9·41	9·22	9·31
Histidine-N	6·45	6·12	7·17	6·47	6·29	6·82	6·55
Lysine-N	9·20	9·37	9·39	9·86	9·29	9·62	9·46
Total N of filtrate	63·30	62·25	61·00	62·70	62·78	61·85	62·31
Amino-N of filtrate	56·90	55·34	54·06	55·46	56·12	54·76	55·44
Non-amino-N of filtrate	6·40	6·91	6·94	7·24	6·66	7·09	6·87
Sum	100·95	100·43	99·94	100·45	100·69	100·19	100·44

The differences between the results for the four samples are in no case greater than would normally be expected between duplicate analyses of any one preparation of caseinogen, so that one may reasonably conclude that, so far as the protein part of the molecule is concerned, the caseinogen of colostrum is identical with that of milk.

GLOBULIN.

Hammarsten seems to have been the first to suggest the presence of a globulin in milk, and a substance with globulin-like characteristics was indeed isolated from milk by Sebelien [1885]. He expressed a doubt, however, whether this substance could be regarded as a true globulin.

Halliburton [1890] on repeating Sebelien's work obtained a small quantity of a protein preparation, which, however, he maintained was not a globulin but merely lactalbumin. As a result of further investigation he arrived at the conclusion that milk does not contain a globulin.

There can be no doubt that this conclusion is erroneous. Not only do biological tests point clearly to the presence of a globulin in milk, but we have succeeded in obtaining from milk small quantities of material which, as the results in Table II indicate, is in every way identical with the undoubted globulin prepared from colostrum.

In the case of the preparation from colostrum the total globulin was resolved into pseudoglobulin and euglobulin by dialysis, but owing to the small quantities involved this was not feasible in the case of the preparation from milk.

TABLE II. *Summary of the*
of Globulin in Per-

	From colostrum			
	Cow 2AI		Cow 10AI	
	(5) Eu globulin %	(6) Pseudo-globulin %	(7) Eu-globulin %	(8) Pseudo-globulin %
Ammonia-N	7·28	7·19	6·92	7·33
Melanin-N	2·00	2·03	2·40	2·20
Total N of bases ...	24·95	24·57	24·52	23·90
Cystine-N	2·00	1·83	2·04	1·81
Arginine-N	10·93	10·70	10·69	10·41
Histidine-N	3·87	4·16	3·68	3·25
Lysine-N	8·15	7·88	8·11	8·43
Total N of filtrate ...	65·31	65·82	63·97	64·57
Amino-N of filtrate ...	64·46	64·49	63·08	63·31
Non-amino-N of filtrate	0·85	1·33	0·89	1·26
Sum	99·54	99·61	97·81	98·00

From Table II it is clear that the total globulin of milk is identical in composition with that of colostrum. Further, it is obvious that, so far as the protein part of their molecules is concerned, eu-lactoglobulin and pseudo-lactoglobulin are identical. This is in complete accord with the similar observations of Hartley [1914] on euglobulin and pseudoglobulin from ox-serum, and lends further support to the hypothesis of Chick [1914] that euglobulins are protein-lipoid complexes resulting from the interaction and mutual precipitation of the colloidal solution of pseudoglobulin with a lipoid emulsion. That differences do exist between the pseudoglobulin and euglobulin prepared from ox-serum is clear from the work of Hardy [1905] and Haslam [1913], but Hartley's results show that this difference is limited to the non-protein ingredients of the preparations, as do our results for the parallel, if not identical, case of the lactoglobulin preparations.

ALBUMIN.

The results of analysis of three samples of albumin from colostrum and two samples from milk are summarised in Table III.

They show clearly that the lactalbumin of colostrum is identical with that of milk, and thus substantiate the indications of biological tests.

Corrected Analyses of Different Preparations
centages of the Total Nitrogen.

From milk			Average				
Cow 2AI		Cow 10AI	From colostrum			From milk	
(9) Total globulin %	(10) Total globulin %	(11) Total globulin %	Eu-globulin (5 and 7) %	Pseudo-globulin (6 and 8) %	Total globulin (5, 6, 7 and 8) %	Total globulin (9, 10 and 11) %	Average of all seven samples %
8·03	8·04	8·22	7·10	7·26	7·18	8·10	7·57
2·23	1·95	2·30	2·20	2·12	2·16	2·16	2·16
25·80	26·82	25·99	24·74	24·24	24·49	26·20	25·23
2·01	2·00	1·59	2·02	1·82	1·92	1·87	1·90
10·59	11·30	10·92	10·81	10·56	10·68	10·94	10·79
3·92	4·13	4·68	3·78	3·70	3·74	4·24	3·96
9·28	9·39	8·80	8·13	8·16	8·15	9·16	8·58
62·89	62·54	63·55	64·64	65·20	64·92	63·00	64·10
61·39	61·52	62·52	63·77	63·90	63·83	61·81	62·97
1·50	1·02	1·03	0·87	1·30	1·09	1·19	1·13
98·95	99·35	100·06	98·68	98·81	98·74	99·46	99·06

TABLE III.

Summary of the Corrected Analyses of Different Preparations of Albumin in Percentages of Total Nitrogen.

	From colostrum			From milk		Average		Average of all five samples %
	Cow 2AI							
	1st sample (12) %	2nd sample (13) %	Cow 10AI (14) %	Cow 2AI (15) %	Cow 10AI (16) %	Colostrum (12, 13 and 14) %	Milk (15 and 16) %	
Ammonia-N	7·92	7·79	8·50	7·75	7·69	8·07	7·72	7·93
Melanin-N	1·97	1·88	1·95	1·50	1·80	1·93	1·65	1·82
Total N of bases ...	26·30	26·83	26·70	26·92	26·87	26·61	26·90	26·72
Cystine-N	1·90	2·32	2·00	2·49	2·19	2·07	2·34	2·18
Arginine-N	7·52	7·60	8·13	7·10	7·44	7·75	7·27	7·56
Histidine-N	4·41	4·61	4·78	4·20	4·21	4·60	4·20	4·44
Lysine-N	12·47	12·30	11·79	13·13	13·03	12·19	13·08	12·54
Total N of filtrate ...	63·06	61·40	62·12	62·89	62·97	62·19	62·93	62·49
Amino-N of filtrate ...	59·07	58·56	59·77	60·71	61·10	59·13	60·91	59·84
Non-amino-N of filtrate	3·99	2·84	2·35	2·18	1·87	3·06	2·03	2·65
Sum	99·25	97·90	99·27	99·06	99·33	98·80	99·20	98·96

Comparison of the Three Proteins.

In view of the divergent views which have been expressed from time to time as to the inter-relation of the proteins of milk it is of interest to compare the results of analysis, for which purpose Table IV gives a convenient summary.

TABLE IV.

	Caseinogen %	Lactoglobulin %	Lactalbumin %
Ammonia-N	10·25	7·57	7·93
Melanin-N	1·26	2·16	1·82
Total N of bases ...	26·62	25·23	26·72
Cystine-N	1·30	1·90	2·18
Arginine-N	9·31	10·79	7·56
Histidine-N	6·55	3·96	4·44
Lysine-N	9·46	8·58	12·54
Total N of filtrate ...	62·31	64·10	62·49
Amino-N of filtrate ...	55·44	62·97	59·84
Non-amino-N of filtrate	6·87	1·13	2·65

These results are conclusive as to the distinct chemical individuality of each of the three proteins. Thus caseinogen is sharply characterised by its relatively high proportion of ammonia-N, histidine-N and "non-amino-N of filtrate" (*i.e.* of proline-N + oxyproline-N + tryptophane-N) and relatively low proportion of melanin-N, cystine-N and α-mono-amino-acids ("amino-N of filtrate"). The arginine and lysine figures for caseinogen also mark it off as a separate protein.

Similarly the lactoglobulin is sharply differentiated from the lactalbumin by its lower content of lysine and its higher content of arginine. This large difference in lysine-content is in agreement with the observations of Hartley on the analogous proteins of ox-serum and horse-serum. The higher cystine-content of lactalbumin is in agreement with the sulphur-content of the two proteins. Thus, in lactalbumin from milk Sebelien found 1·73 % S, whilst in lactoglobulin from colostrum Tiemann [1898] found only 1·24 % S.

The foregoing conclusions as to the distinct chemical individuality of the three milk proteins are in complete accord with conclusions as to the biological differentiation of the three proteins which have been arrived at by the precipitin method [Hamburger, 1901], the method of complement deviation [Bauer and Engel, 1911] and anaphylactic methods [Besredka, 1909; Heuner, 1911].

TABLE V.

Showing the Similarity in Composition of the Globulins of Cow's Colostrum, Cow's Milk and Ox-Serum.

	Total globulin		Euglobulin		Pseudoglobulin		Average of all samples	
	From milk (average of three samples) (9, 10, 11) %	From ox-serum (one sample) (Hartley) %	From colostrum (average of two samples) (5, 7) %	From ox-serum (average of two samples) (Hartley) %	From colostrum (average of two samples) (6, 8) %	From ox-serum (one sample) (Hartley %	From milk and colostrum (seven samples) (5–11) %	From ox-serum (four samples) (Hartley) %
Ammonia-N	8·10	7·7	7·10	8·65	7·26	7·5	7·57	8·13
Melanin-N	2·16	2·0	2·20	2·25	2·12	1·9	2·16	2·10
Total N of bases ...	26·20	28·2	24·74	27·65	24·24	27·1	25·23	27·65
Cystine-N	1·87	2·0	2·02	1·7	1·82	1·9	1·90	1·82
Arginine-N	10·94	10·9	10·81	11·65	10·56	10·8	10·79	11·25
Histidine-N	4·24	6·3	3·78	5·15	3·70	4·8	3·96	5·35
Lysine-N	9·16	9·0	8·13	9·15	8·16	9·6	8·58	9·23
Total N of filtrate ...	63·00	62·0	64·64	60·05	65·20	63·3	64·10	61·35
Amino-N of filtrate ...	61·81	59·8	63·77	57·95	63·90	61·7	62·97	59·35
Non-amino-N of filtrate	1·19	2·2	0·87	2·1	1·30	1·6	1·13	2·00
Sum	99·46	99·9	98·68	98·60	98·81	99·8	99·06	99·23

Comparison with the Proteins of Ox-Serum.

The question of the relationship of the proteins of milk to those of the blood is one of obvious interest and has already received considerable attention. The earlier investigators, relying upon ultimate chemical analysis and methods of precipitation, concluded that the albumins of milk and of blood were identical. Later workers, using the biological methods elaborated by workers on immunity, have confirmed this conclusion and extended it further to the globulins [Bauer and Engel, 1911].

Our results obtained with lactoglobulin and lactalbumin, when taken in conjunction with those obtained by Hartley [1914] with ox-serum globulin and ox-serum albumin, whilst confirming the identity of the globulins, do not harmonise with the foregoing conclusions, so far as the albumins are concerned. For convenience of comparison we have assembled the data necessary for the comparison in Tables V and VI.

(*a*) *Comparison of Globulins.*

Table V contains a summary of the average results obtained by Hartley and by us in the analysis of the respective globulins.

Comparison of these results shows a striking resemblance throughout between the different preparations. The greatest variation is seen in the histidine values, but, as pointed out earlier, these are subject to relatively greater errors than any other of the values. If due allowance be made for unavoidable experimental error, and for the varying personal error of the different observers, there can be little doubt that these results, so far as they go, indicate that the respective globulins, whether total globulin, pseudo-globulin, or euglobulin, of colostrum and milk are identical with those of ox-serum.

(*b*) *Comparison of Albumins.*

The average results obtained by us for lactalbumin are summarised in Table VI, together with Hartley's analysis of serum albumin. This table also includes in the last column the average of three analyses by van Slyke [1915] of a sample of lactalbumin obtained from milk by precipitation of the caseinogen by acetic acid and coagulation of the lactalbumin by heating. This preparation would obviously be contaminated with the small amount of lactoglobulin present in the milk, but the analysis serves as a useful check upon our results obtained with purer preparations.

TABLE VI.

Showing the Differences in Composition of the Albumins of Cow's Milk and Ox-Serum.

	Lactalbumin			Serum-albumin	
	From colostrum (average of three samples, 12–14)	From milk (average of two samples, 15, 16)	Average of all five samples (12–16)	One sample (Hartley)	Lactalbumin Average of three samples (van Slyke)
	%	%	%	%	%
Ammonia-N	8·07	7·72	7·93	5·8	8·57
Melanin-N	1·93	1·65	1·82	1·1	2·32
Total N of bases ...	26·61	26·90	26·72	36·9	25·31
Cystine-N	2·07	2·34	2·18	3·5	1·30
Arginine-N	7·75	7·27	7·56	10·4	7·20
Histidine-N	4·60	4·20	4·44	6·7	4·57
Lysine-N	12·19	13·08	12·54	16·3	12·24
Total N of filtrate ...	62·19	62·93	62·49	56·5	63·50
Amino-N of filtrate ...	59·13	60·91	59·84	54·2	61·53
Non-amino-N of filtrate	3·06	2·03	2·65	2·3	1·97
Sum	98·80	99·20	98·96	100·3	99·70

Comparison of the results reveals marked differences between our lactalbumin and Hartley's serum albumin. This is strikingly manifest in every item of the analyses, but notably in the basic nitrogenous constituents. The reliability of our results for lactalbumin may be gauged from the separate analyses set out in Table III, and further from the reasonably close concordance with van Slyke's results. On the other hand, we have for serum albumin only the one analysis by Hartley, although his results are substantially confirmed by those obtained by him with albumin from horse-serum.

As already mentioned most of the older chemical work and the more recent biological work has pointed to the identity of lactalbumin with serum albumin, but observations that do not harmonise with this conclusion have not been lacking.

Thus Sebelien [1885] found that albumin whether prepared from colostrum or milk had a specific rotatory power of $[\alpha]_D = -36°$ to $-37°$, whilst serum albumin isolated by a similar method from ox-blood gave $[\alpha]_D = -62{\cdot}6°$. Further, Halliburton [1890] showed that lactalbumin differed from serum albumin in its behaviour on heat-coagulation, and in its precipitability by certain neutral salts.

There is obvious need for further investigation here, but it is clear that biological tests alone will not suffice for the purpose. With the material at our disposal we propose to investigate this point more closely.

EXPERIMENTAL DETAILS.

Preparations of the Proteins.

Summary. *Caseinogen* was prepared by precipitation from the diluted colostrum or milk with saturated potash alum solution (Schlossmann); *Total Globulin* by saturation of the neutralised filtrate from the caseinogen with anhydrous magnesium sulphate; the two globulins were separated by dialysis; *Albumin* was obtained from the filtrate from the total globulin by saturation with anhydrous sodium sulphate at 40° C. (Pinkus). Ammonium sulphate was not used as a precipitant for the globulin and albumin in order to avoid the possibility of adsorption of extraneous nitrogen by the proteins.

Caseinogen. Freshly drawn colostrum was diluted with five times its volume of *distilled* water[1] and heated to 40° in order to produce a more satisfactory settling of the caseinogen. Saturated potash alum solution was then added in sufficient amount to produce complete precipitation of the caseinogen. In the case of milk, dilution with only three times its volume of water was necessary and less alum solution was also needed. After settling for half an hour, the clear supernatant liquid was syphoned off, the residuum of caseinogen and fat strained on muslin, washed three times by stirring vigorously with water, and the filtered washings added to the main bulk of liquid, for the preparation of globulin and albumin.

Before proceeding with the purification of the caseinogen, it was necessary to free it from the large amount of fat carried down with it on precipitation. This was done at first by extracting the semi-solid mass with alcohol and then with ether. It was found preferable, however, to extract the fat with ether from the solution of caseinogen in alkali. For this purpose the caseinogen, suspended in distilled water, which was kept well stirred, was dissolved in the minimum amount of 1% soda and the solution extracted with ether. The ethereal layer carried with it most of the insoluble matter, and filtration of the solution of caseinogen in alkali was thereby rendered much easier. The filtered solution was diluted with distilled water and the caseinogen reprecipitated with 1% acetic acid, added with constant stirring. After settling, the supernatant liquid was syphoned off and the caseinogen washed by sedimentation. This process of dissolving in alkali and reprecipitating was repeated five times. The final solution in alkali was filtered clear through filter paper pulp, diluted, and precipitated with the minimum amount of 1% hydrochloric acid. After washing and draining, the caseinogen was dried by grinding with alcohol, and finally with ether. The final product (air-dried) was a perfectly white, dusty powder.

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[1] It is essential, in order to produce a perfectly white sample of caseinogen, that all dilutions should be done with distilled water. The use of tap water, especially if of peaty origin, causes the production of a brownish coloured product.

Globulin. The filtrate from the caseinogen preparation was neutralised with dilute soda, and saturated with anhydrous magnesium sulphate, thus obviating the large increase in volume which occurs if the crystalline salt is used. The precipitated globulin was filtered off on large Buchner funnels and the filtrate used for the isolation of albumin. The crude globulin was dissolved in distilled water to which a little magnesium sulphate had been added, the solution filtered through pulp, saturated with magnesium sulphate, and the globulin filtered off. This reprecipitation was repeated four times at the same volume each time, and the filtrate in each case was added to the albumin preparation. The final solution of globulin was then dialysed in large sausage-skin dialysers for six days in running water, and then in relays of distilled water until free from sulphates. The globulin was thus separated into two fractions; euglobulin, insoluble in distilled water, and pseudoglobulin, soluble in distilled water.

Euglobulin. This was filtered from the soluble pseudoglobulin, dissolved in the minimum amount of 0·6% sodium chloride solution, filtered, and reprecipitated by diluting the solution with ten times its volume of distilled water. The precipitate was allowed to settle, washed with distilled water, and finally dried with alcohol and ether, thus giving an almost white powder.

Pseudoglobulin. The filtered solution of pseudoglobulin in water was precipitated with alcohol, filtered, and the protein dried with alcohol and ether, giving a perfectly white powder.

Albumin. This protein was isolated and purified by Pinkus' method [1901]. The combined filtrates from the globulin preparation were heated to 40°, saturated with anhydrous sodium sulphate (free from chlorides) and allowed to stand at this temperature for a few hours. The precipitated albumin was then filtered off through a warm jacketed filter, care being taken to prevent the salt from crystallising. The crude albumin was dissolved in cold water, filtered, and the solution saturated with magnesium sulphate. The slight precipitate, probably globulin, was filtered off, and the filtrate saturated with anhydrous sodium sulphate at 40° as before. This process was repeated four times, saturation with magnesium sulphate being from the same volume each time. The last saturation with magnesium sulphate gave no precipitate. The final precipitate of lactalbumin was dissolved in water and the filtered solution precipitated with alcohol, the protein being finally dried with alcohol and ether, and gave a white, light powder, readily soluble in water.

In the preparation of the proteins from colostrum the first three milkings after calving from two cows (2AI and 10AI) were taken, and the proteins isolated from each of these separately. Had any appreciable differences between the respective proteins of colostrum and milk been found, it was proposed to analyse separately each sample of protein obtained. Since, however, no such differences could be detected, only the preparations from the first sample of colostrum after calving were analysed.

The proteins from milk were prepared from samples taken from the same cows three months after calving, when it could be safely assumed that the milk had become quite normal. The method of isolation was the same as for colostrum. It has been remarked, however, that in the purification of the albumin from colostrum, when the solution of the albumin was saturated with magnesium sulphate, it gave in the first two or three reprecipitations a slight precipitate with magnesium sulphate, which was supposed to be a globulin. In working up the colostrum these small amounts of globulin were rejected, an ample yield being otherwise obtained. In the case of milk, however, the combined precipitates obtained by saturation with magnesium sulphate in the purification of the albumin, were added to the main bulk of the globulin precipitate, and the whole purified by reprecipitation with magnesium sulphate six times. That it was really a part of the globulin which remained dissolved in the albumin solution is clear from the analyses, and the recovery of these portions probably accounts for the fact that a much bigger yield of globulin was obtained from milk than has hitherto been described. Twenty litres of milk from each cow were worked up separately, but only a portion of the caseinogen was purified. The following amounts of lactoglobulin and lactalbumin were isolated. From 20 litres of milk in each case

Cow 2AI gave 7·28 g. lactoglobulin and 43·0 g. lactalbumin.

Cow 10AI gave 4·54 g. lactoglobulin and 35·4 g. lactalbumin.

Owing to the difficulty of obtaining more than small quantities of the globulin in milk, and since preliminary analyses had pointed to the fact that euglobulin and pseudoglobulin from colostrum are indistinguishable by the method of analysis used, the samples of globulin obtained from milk were not fractionated into eu- and pseudoglobulin, so that in this case data for total globulin only are recorded in the tables.

METHODS OF ANALYSIS.

Van Slyke's method of protein analysis was used throughout these experiments. For details the original paper must be consulted.

In order that the analyses might be strictly comparable the conditions of analysis of each protein were made as nearly the same as possible. Whenever the amount available permitted it, 3·5 g. of the protein, air dried, were used for an analysis, the lowest amount used (analysis 10, Table VII) being 2·5 g. In all cases the protein was hydrolysed by heating with thirty-five times its weight of 20 % hydrochloric acid for 48 hours at 100°, using flasks with air-condensers ground into the necks. The resultant mixture was evaporated *in vacuo* to remove as much HCl as possible and was then made up to 100 cc., and the following estimations were made:

(i) *Total N* estimated (Kjeldahl) on two separate portions of 10 cc. each.

(ii) 75 cc. were taken for the subsequent analysis and gave

(*a*) *Ammonia-N* in the 75 cc.

(*b*) *Melanin-N* in the 75 cc.

After removal of the ammonia and melanin the bases were precipitated with phosphotungstic acid, and after removal of the phosphotungstic acid with baryta, the solution of the bases was made up to 100 cc., and the following estimations made on this solution:

(*c*) *Cystine-N* in 20 cc. of the solution of the bases.

(*d*) *Arginine-N* in 50 cc. of the solution of the bases.

(*e*) *Residual-N* (*i.e.* half the arginine-N and total N of cystine, histidine, and lysine) in the same 50 cc. of solution as (*d*), so that (*d*) + (*e*) gave the total N of the bases.

(*f*) *Amino-N of the bases* in 2 cc. of the solution: done in triplicate.

The filtrate from the phosphotungstic acid precipitate of the bases was made up to 200 cc. and the following estimations were made on this solution:

(*g*) *Total N of the filtrate* in 50 cc.: done in duplicate.

(*h*) *Amino-N of the filtrate*; 15 cc. were diluted to 25 cc. and the amino-N estimated in 2 cc.: done in triplicate.

(*i*) *Non-amino-N of the filtrate,* was calculated by taking the differences of (*g*) and (*h*).

Table VII contains a record of the whole of the analytical observations performed on each protein, as described in the above scheme, whilst Table VIII gives a summary of the whole of the results obtained.

			Amino-N of filtrate				Total N of filtrate		
	Nature of preparati	t. N per 75 cc.	cc. gas per 2 cc.	$t°$	p mm.	g. N per 75 cc.	cc. thio. per 50 cc.	g. N per 75 cc.	Total N recovered g. per 75 cc.
1.	Caseinogen from colost	0·04774	2·375 2·363 2·388	19·0 18·2 18·8	765·7 765·1 764·4	0·2069	42·04 42·11	0·2345	0·3579
2.	Caseinogen from colost	0·04766	2·265 2·255 2·252	18·6 18·1 18·0	767·3 767·3 767·3	0·1966	40·50 40·40	0·2254	0·3473
3.	Caseinogen from milk.	0·05312	2·347 2·380 2·355	13·1 14·2 14·1	761·0 761·0 761·0	0·2094	43·09 43·06	0·2405	0·3774
4.	Caseinogen from milk.	0·05004	2·332 2·336 2·335	18·8 19·2 19·0	768·2 768·0 768·0	0·2035	42·03 42·04	0·2343	0·3592
5.	Euglobulin from colost	0·05041	2·900 2·905 2·880	15·0 15·0 13·5	753·3 753·2 755·0	0·2580	47·41 47·39	0·2663	0·3905
6.	Pseudoglobulin from co	0·04907	2·910 2·905 2·915	14·7 15·0 14·6	752·6 754·7 754·7	0·2592	47·96 47·91	0·2693	0·3923
7.	Euglobulin from colost	0·05129	2·900 2·902 2·880	15·0 15·0 15·0	756·2 756·2 761·2	0·2588	47·58 47·58	0·2673	0·3932
8.	Pseudoglobulin from co	0·05127	2·900 2·900 2·890	15·0 13·2 13·0	760·6 762·0 762·0	0·2620	48·45 48·40	0·2720	0·3974
9.	Total globulin from mi	0·05286	2·713 2·697 2·718	15·0 14·5 14·8	751·7 751·4 750·8	0·2389	44·69 44·83	0·2495	0·3767
10.	Total globulin from mi	0·03637	1·965 1·975 1·965	13·8 14·0 13·0	750·0 750·0 755·8	0·1689	31·67 31·66	0·1765	0·2644
11.	Total globulin from mi	0·04196	2·355 2·355 2·360	15·4 14·1 14·7	754·4 754·4 754·0	0·2059	38·42 38·44	0·2141	0·3212
12.	Albumin from colostru	0·04802	2·142 2·128 2·126	19·2 19·0 19·0	763·4 763·1 763·1	0·1826	35·75 35·83	0·1995	0·2980
13.	Albumin from colostru (Duplicate of 12.)	0·04877	2·095 2·105 2·105	17·6 17·9 17·9	760·7 760·2 760·2	0·1802	34·72 34·72	0·1936	0·2926
14.	Albumin from colostru	0·05624	2·478 2·476 2·482	17·5 16·9 17·4	758·0 757·8 757·3	0·2161	41·17 41·11	0·2293	0·3502
15.	Albumin from milk.	0·06222	2·565 2·578 2·568	16·8 16·7 17·5	751·2 751·2 751·2	0·2228	42·26 42·26	0·2355	0·3550
16.	Albumin from milk.	0·06022	2·540 2·535 2·530	16·0 15·8 15·6	751·5 752·6 753·2	0·2214	41·82 41·79	0·2329	0·3515

TABLE VIII. *Results of Analyses expressed in Percentages of the Total Nitrogen.*

(*a*) *Uncorrected.* (*b*) *Corrected for solubility of the Phosphotungstates of Bases.*

Protein analysed		Ammonia N	Melanin N	Total N of bases	Cystine N	Arginine N	Histidine N	Lysine N	Total N of filtrate	Amino N of filtrate	Non-amino N of filtrate	Sum
1. Caseinogen (colostrum). Cow 2AI	(*a*)	10·42	1·29	23·09	0·60	8·05	5·38	9·06	66·15	58·38	7·77	100·95
	(*b*)	10·42	1·29	25·94	1·34	8·95	6·45	9·20	63·30	56·90	6·40	
2. Caseinogen (colostrum). Cow 10AI	(*a*)	10·07	1·35	23·83	0·65	8·94	5·02	9·22	65·18	56·86	8·32	100·43
	(*b*)	10·07	1·35	26·76	1·40	9·87	6·12	9·37	62·25	55·34	6·91	
3. Caseinogen (milk). Cow 2AI	(*a*)	10·15	1·27	24·84	0·54	8·89	6·16	9·25	63·68	55·46	8·22	99·94
	(*b*)	10·15	1·27	27·52	1·23	9·73	7·17	9·39	61·00	54·06	6·94	
4. Caseinogen (milk). Cow 10AI	(*a*)	10·35	1·12	23·46	0·51	7·82	5·41	9·72	65·52	56·93	8·59	100·45
	(*b*)	10·35	1·12	26·28	1·24	8·71	6·47	9·86	62·70	55·46	7·24	
5. Euglobulin (colostrum). Cow 2AI	(*a*)	7·28	2·00	22·39	1·34	10·12	2·91	8·02	67·89	65·79	2·10	99·54
	(*b*)	7·28	2·00	24·95	2·00	10·93	3·87	8·15	65·31	64·46	0·85	
6. Pseudoglobulin (colostrum). Cow 2AI	(*a*)	7·19	2·03	22·01	1·17	9·89	3·20	7·75	68·36	65·83	2·53	99·61
	(*b*)	7·19	2·03	24·57	1·83	10·70	4·16	7·88	65·82	64·49	1·33	
7. Euglobulin (colostrum). Cow 10AI	(*a*)	6·92	2·40	22·01	1·39	9·90	2·73	7·99	66·48	64·39	2·09	97·81
	(*b*)	6·92	2·40	24·52	2·04	10·69	3·68	8·11	63·97	63·08	0·89	
8. Pseudoglobulin (colostrum). Cow 10AI	(*a*)	7·33	2·20	21·40	1·16	9·62	2·31	8·31	67·07	64·60	2·47	98·00
	(*b*)	7·33	2·20	23·90	1·81	10·41	3·25	8·43	64·57	63·31	1·26	
9. Total globulin (milk). Cow 2AI	(*a*)	8·03	2·23	23·15	1·32	9·75	2·92	9·16	65·52	62·75	2·77	98·95
	(*b*)	8·03	2·23	25·80	2·01	10·59	3·92	9·28	62·89	61·39	1·50	
10. Total globulin (milk). Cow 2AI	(*a*)	8·04	1·95	23·03	1·02	10·09	2·70	9·22	66·33	63·48	2·85	99·35
	(*b*)	8·04	1·95	26·82	2·00	11·30	4·13	9·39	62·54	61·52	1·02	
11. Total globulin (milk). Cow 10AI	(*a*)	8·22	2·30	22·85	0·78	9·93	3·49	8·65	66·71	64·15	2·56	100·06
	(*b*)	8·22	2·30	25·99	1·59	10·92	4·68	8·80	63·55	62·52	1·03	
12. Albumin (colostrum). Cow 2AI	(*a*)	7·92	1·97	22·93	1·03	6·46	3·14	12·30	66·40	60·78	5·62	99·25
	(*b*)	7·92	1·97	26·30	1·90	7·52	4·41	12·47	63·06	59·07	3·99	
13. Albumin (colostrum). Cow 2AI	(*a*)	7·79	1·88	23·46	1·45	6·53	3·34	12·14	64·77	60·30	4·47	97·90
	(*b*)	7·79	1·88	26·83	2·32	7·60	4·61	12·30	61·40	58·56	2·84	
14. Albumin (colostrum). Cow 10AI	(*a*)	8·50	1·95	23·83	1·26	7·22	3·70	11·65	65·00	61·25	3·75	99·27
	(*b*)	8·50	1·95	26·70	2·00	8·13	4·78	11·79	62·12	59·77	2·35	
15. Albumin (milk). Cow 2AI	(*a*)	7·75	1·50	24·11	1·77	6·21	3·14	12·99	65·70	62·18	3·52	99·06
	(*b*)	7·75	1·50	26·92	2·49	7·10	4·20	13·13	62·89	60·71	2·18	
16. Albumin (milk). Cow 10AI	(*a*)	7·69	1·80	24·02	1·45	6·54	3·14	12·89	65·83	62·56	3·27	99·33
	(*b*)	7·69	1·80	26·87	2·19	7·44	4·21	13·03	62·97	61·10	1·87	

Observations on the Method of Analysis.

(i) In all estimations of nitrogen by the Kjeldahl method the excess of acid used for neutralising the ammonia was determined by adding to the liquid to be titrated a little solid potassium iodide and then a few cc. of a saturated solution of normal potassium iodate. The iodine liberated by the free acid present was then estimated by titration with standard (N/10) sodium thiosulphate, using starch paste as indicator. A sharper end-point was thereby obtained than with the ordinary acidimetric method of titration. The strength of the thiosulphate solution was determined at short intervals by checking it against the standard acid used, and by titration with weighed amounts of pure iodine.

(ii) The filtration of the gelatinous melanin precipitate produced by the lime is very slow if carried out on an ordinary filter paper. It was found to be performed more expeditiously and the precipitate needed less washing to free it from chlorides by filtering by suction. In this, as in the filtration and washing of the phosphotungstates of the bases, the filtering tube as described by Irvine [1915] was used. A double gravimetric filter paper was fitted into the Buchner funnel, and a very slightly reduced pressure maintained all the time in the tube. The precipitate, if not allowed to become dry, when it cracks, is thus easily and quickly washed free from chlorides.

(iii) In the estimation of amino-nitrogen, van Slyke's micro-apparatus [1914] was used throughout. By this means all the estimations could be done in triplicate, and thus with greater accuracy than with the large apparatus. Increased accuracy was further obtained by enclosing the gas measuring burette in a glass jacket through which cold water of known temperature was slowly circulated, so that the volume of gas could be conveniently read off without any disturbance of its temperature. By this means, and with the aid of a lens in taking readings—the burette being of 5 cc. capacity with divisions of 0·01 cc.—it was found possible to read the volume of the gas to 0·002 cc. and to obtain triplicates agreeing within 0·01 cc. The liquid to be analysed was measured from a calibrated 2 cc. pipette and washed into the apparatus with a few drops of distilled water.

(iv) *Corrections for Reagents.* The following were the corrections determined for the impurities in the reagents. All the readings in Table VII have been corrected except the amino-nitrogen estimations.

Total N. 0·16 cc. thio. *Ammonia-N* nil. *Melanin-N.* 0·24 cc. thio.
Cystine-N. 0·0023 g. $BaSO_4$. *Arginine-N.* 0·16 cc. thio.

Residual N of bases 0·18 cc. thio. *Amino-N of bases* 0·188 mgm. N.

Total N of filtrate 0·18 cc. thio. *Amino-N of filtrate* 0·129 mgm. N.

(v) *Corrections for the solubility of the phosphotungstates of the bases.* The phosphotungstates of the bases are slightly soluble in the liquid from which they are precipitated. In order to correct for this error the bases were always precipitated from the same volume of solution (200 cc.) and the corrections as determined by van Slyke [1911, p. 32] applied.

The analyses given in Tables I–VI have all been corrected in this way, while Table VIII contains a summary of the analyses, expressed as percentages of the total nitrogen, before and after correction.

SUMMARY.

The chief proteins of cow's colostrum and cow's milk were isolated, and analysed by the method of van Slyke, with the following results.

1. Caseinogen, total lactoglobulin, and lactalbumin are sharply differentiated, distinct proteins, and have respectively the same composition whether prepared from colostrum or normal milk.

2. The presence in milk of a globulin in small quantities (0·03 % isolated) was proved.

3. Eu-lactoglobulin and pseudo-lactoglobulin are identical so far as the protein part of their molecules is concerned.

4. Lactoglobulin from either colostrum or milk is very closely allied to, and is probably identical with, serum globulin from ox-blood.

5. Lactalbumin from either colostrum or milk is very different in composition from serum albumin from ox-blood.

A considerable portion of the work described in this communication has been carried out in the Animal Nutrition Research Laboratory of the School of Agriculture, Cambridge, and we would take this opportunity of acknowledging our indebtedness to Professors Wood and Hopkins for the facilities kindly accorded for the purpose.

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XXXVI. THE ACTION OF HYPOCHLORITES AND ALLIED SUBSTANCES ON PROTEINS, AND THEIR BEHAVIOUR ON INJECTION.

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The recent work of Lorrain Smith [1915, 1, 2], Dakin [1915, 1, 2, 1916] and others has directed attention to the value of substances containing chlorine in "available" form as germicidal agents in the treatment of infected wounds. Although such substances have long been recognised as powerfully germicidal, they were not previously made use of to any large extent owing to the decrease in their efficiency in the presence of albuminous material. When small quantities of such a fluid as blood serum are added to hypochlorite solutions there is, even at comparatively low temperatures, an initial rapid lowering in the "available" chlorine value followed by a much slower rate of loss. The initial rapid loss is evidently due to oxidation changes and to the passage of chlorine into the cyclic rings of such protein derivatives as tyrosine etc., the hydrogen attached to the carbon being replaced. Part of the chlorine however, especially at low temperatures, replaces the hydrogen attached to nitrogen in various primary and secondary amine groups, forming compounds containing $.N:Cl_2$ or $:N.Cl$ groups. As the chlorine in these groups is still in active form, such compounds, if formed under the ordinary conditions of wound treatment or after intravascular injection, would probably constitute valuable germicidal agents. The significance and value of these chloramino-derivatives of protein must depend in the first place on the rate of formation and concentration of these bodies existing in the wound or in the blood, and, in the second place, on the rate at which these compounds lose their available chlorine either by hydrolysis or by transference to the cyclic rings.

In order to gain some knowledge of the changes which hypochlorites and aromatic substances containing chloramine groups undergo in the presence of proteins, it is necessary to carry out the tests at the temperature to which these bodies would be exposed in wound treatment. A series of experiments was therefore carried out to determine the percentage loss of available chlorine, under the above mentioned conditions, at stated intervals during the reaction. From such a study information may also be obtained as to the order of the chemical reactions concerned, but in this communication very little reference will be made to this part of the subject.

THE ACTION OF HYPOCHLORITES ON ALBUMINOUS SOLUTIONS.

Even extremely weak solutions of free HOCl precipitate albumins but solutions of the hypochlorites in general use have not this effect. At body temperature there is however a rapid disintegration of the protein, nitrogen coming freely off and the amino-acids breaking down into aldehydes or ketones with one carbon atom less than the original acids. The odour of aldehyde formation is very characteristic of the action of hypochlorites on proteins. The rate of change of all hypochlorite solutions in the presence of albumin is approximately the same, and as I was engaged in the study of the action of magnesium hypochlorite as a possibly less irritant antiseptic than sodium or calcium hypochlorite, the majority of the experiments were carried out with this solution.

The method of preparation of $Mg(OCl)_2$ was the following: hypochlorous acid was prepared from bleaching powder and boric acid used in the following proportions, 10 g. bleaching powder, 300 cc. water, and 15–20 g. boric acid. Distillation took place in vacuo and the distillate was collected in a vessel containing an excess of freshly precipitated and washed $Mg(OH)_2$, the receiver being kept in an ice-salt mixture. From 50–100 cc. were distilled over, and the solution so obtained contained 4–5 g. HOCl per litre. These solutions are quite stable and may be kept for at least a month. A similar solution may be conveniently and rapidly prepared by the addition of magnesium sulphate solution to one of bleaching powder, the precipitate being allowed to settle and the fluid siphoned off. Such a solution has been used with advantage as a fairly non-irritant antiseptic by Charles Mayer [1916].

The rate of change of hypochlorite in the presence of albumin was determined in the following way. The hypochlorite solution in a stoppered bottle was brought up to a temperature of 36° in a thermostat which also contained a vessel with blood serum. The "available" chlorine value of the former was estimated and when both solutions had reached the proper temperature, a small fixed quantity of the serum was added to a known volume of the warmed hypochlorite. At definite intervals the available chlorine value of the mixture was determined by running a measured quantity of the mixture into potassium iodide solution acidified with acetic acid and titrating the iodine set free with N/10 thiosulphate. In order to determine the type of the reaction estimations of the chlorine loss were carried out with different concentrations of hypochlorite the quantity of serum remaining constant, and also with hypochlorite solutions of the same strength but with varying quantities of serum.

Before referring to the curves of fall in concentration of active chlorine in the presence of serum, it is necessary to gain some idea of the amount of hypochlorite which can be used up at room temperature by a given amount of protein when an excess of the former is used and when sufficient time is given to complete the reaction. Effront [1905] has calculated that 1 mgm. protein (egg white) uses up 3·055 mgm. active chlorine, or for 1 mgm. nitrogen of the protein 18·629 mgm. chlorine. Using the same method as Effront, I have found that 1 mgm. nitrogen of blood serum uses up 15·87 mgm. $Mg(OCl)_2$. Therefore when 5 cc. serum are taken containing 61 mgm. nitrogen about 0·968 g. $Mg(OCl)_2$ can be used up. 200 cc. $Mg(OCl)_2$ *m*/20 contain 1·2722 g. $Mg(OCl)_2$, so that if 5 cc. serum were added to this solution there would be at the end of the reaction a slight excess of hypochlorite, while with the weaker hypochlorite solutions or with higher protein concentrations, the protein would be in excess[1].

Fig. 1 shows the available chlorine fall when 200 cc. of *m*/20 $Mg(OCl)_2$ (Graph I) and 200 cc. *m*/40 $Mg(OCl)_2$ (Graph II) respectively are mixed with 5 cc. blood serum (containing 0·061 g. nitrogen) the mixture being kept at 36°. Control solutions of $Mg(OCl)_2$ of the same strength without the addition of serum were also kept under the same temperature conditions and the available chlorine determined at the stated intervals. The control solutions showed practically no alteration during the period of the experiment.

The curves show a fall down to 30–35 % of the original value within 15 minutes, but after the first half hour the rate of loss of available chlorine becomes very much slower. The curves are of the same type, slightly the greater fall taking place with the weaker solution. At the end of three hours, the available chlorine value is only slightly over 10 % of the initial concentration and is approximately the same in both cases. The curves show falls which are practically independent of the initial concentration.

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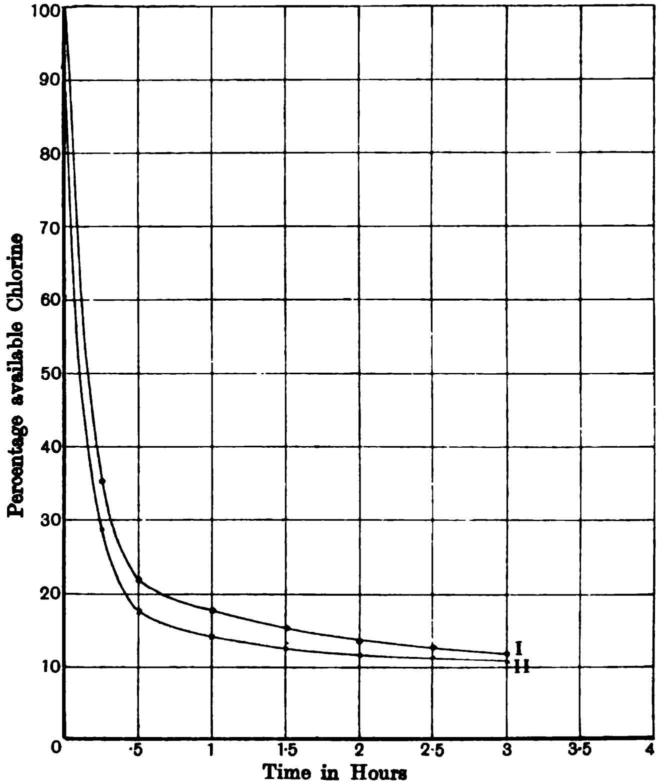
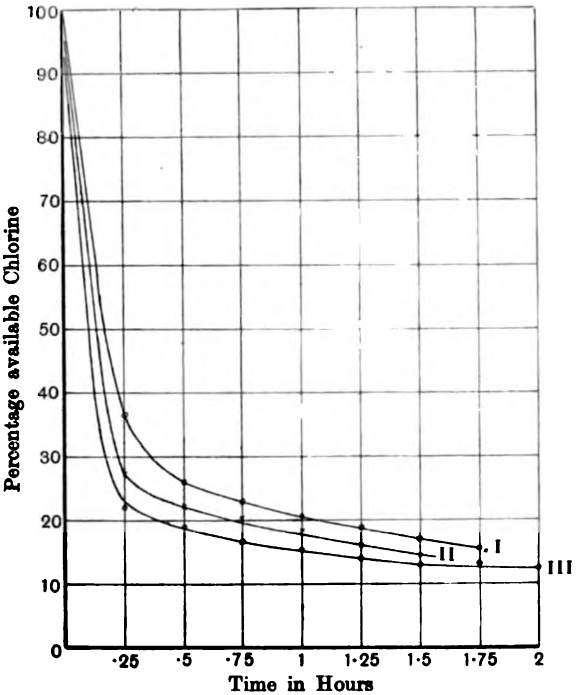
[1] The interesting observations of Dakin [1916] published after the completion of my work show that with higher protein concentrations than I used with hypochlorite solutions, namely from 1 cc. up to 10 cc. serum added to 5 cc. neutral hypochlorite containing 0·0126 g. HOCl, more active chlorine disappears in a given time with the smaller amounts of serum than with the larger.



200 cc. Mg $(OCl)_2$ *m*/20 + 5 cc. Blood Serum = I
" " *m*/40 + " " " = II

Fig. 1.

Fig. 2 shows the effect of varying the protein and keeping the initial hypochlorite concentration the same. Three 200 cc. portions of *m*/20 Mg $(OCl)_2$ were taken and 5 cc. (Graph I) 10 cc. (Graph II) and 20 cc. serum (Graph III) respectively were added to the solutions. The available chlorine value in the first case fell to 37 % of the initial value, in the second to 28 % and in the third to 22 % within 15 minutes of admixture. This primary rapid fall is succeeded by the characteristic slow fall observed in Fig. 1. The types of curve are the same and at the end of 2 hours the active chlorine is about 12–15 % of the initial value in the three solutions. The fall continues slowly during the next 24 hours.



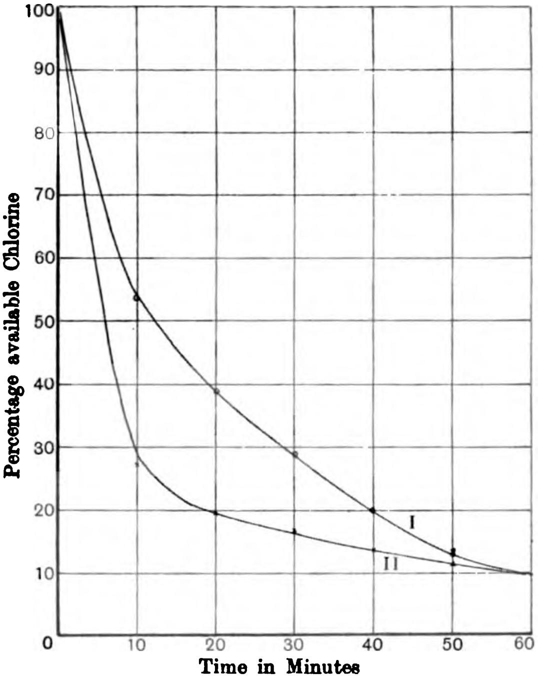
200 cc. Mg $(OCl)_2$ $m/20+$ 5 cc. Serum = I
" " " +10 cc. " = II
" " " +20 cc. " = III

Fig. 2.

In Fig. 3 the fall is plotted out for shorter intervals and for solutions of eusol (Graph I) of a strength which is commonly used as an antiseptic (0·315 % "available" chlorine) and Mg $(OCl)_2$ (0·556 % "available chlorine") (Graph II).

In the case of the former 5 cc. serum were added and in the latter 9 cc.

In the case of Graph I, Fig. 3 apart from the fall during the first 10 minutes, the logarithm of the concentration is practically proportional to the time—a reaction of the first order. At the close of the first hour the chlorine value in both cases is only 10 % of the initial figure. From this point onwards the fall becomes very slow in both cases so that 18 hours later the chlorine value is still 3 % of the initial figure.



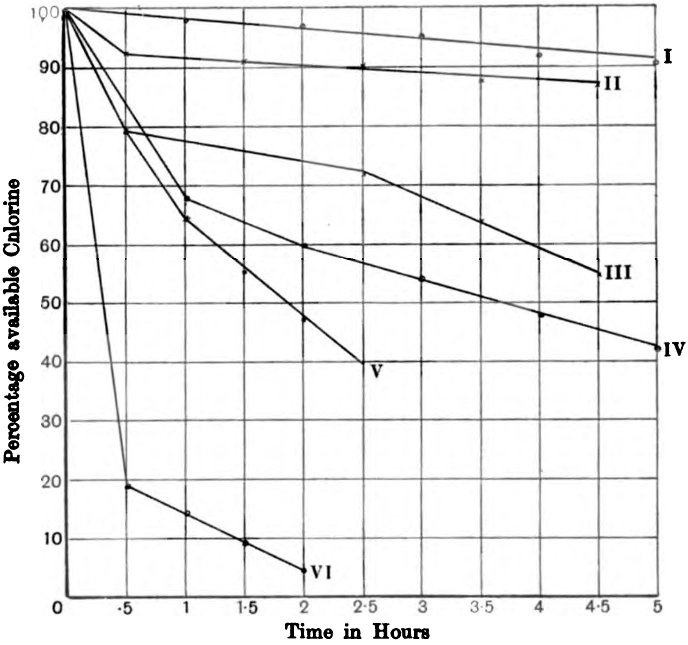
200 cc. "Eusol" (0·315% Chlorine) + 5 cc. Serum = I
„ $Mg(OCl)_2$ (0·556% „) + 9 cc. „ = II

Fig. 3.

THE ACTION OF CHLORAMINE-T ON ALBUMINS.

The valuable work of Dakin and his collaborators [1916] has directed attention to substances containing chlorine attached to nitrogen in primary and secondary amine groups. The chemical characters and bactericidal properties of a number of aromatic chloramines prepared originally by Chattaway have been studied by these investigators. As the action of hypochlorites on albumins probably results, in small part at least, in the formation of bodies of this type, it is probable that from a comparison of the velocity of reaction of such a body as sodio-*p*-toluene-sulphochloroamide ("chloramine-T") in albuminous fluids with the reaction velocity of hypochlorites in the same fluids, one may arrive at some knowledge of the nature of the latter reaction. When chloramine solutions are mixed with varying quantities of blood serum, the reaction is not only a very much slower one but also differs in many other particulars from the hypochlorite-serum reaction. Thus the fall varies directly with the volume of serum added.

The percentage fall in available chlorine when chloramine solutions are mixed with different quantities of serum was determined in the same way as in the hypochlorite-serum mixtures. All the experiments were carried out at 36°. When small quantities of serum, similar to those used in the hypochlorite experiments, are added to chloramine solutions the rate of fall in available chlorine is extremely slow. Fig. 4 shows a series of graphs with varying concentrations of chloramine and albumin:



200 cc. Chloramine-T 1·338 % + 5 cc. Serum = I
,, ,, 1 % + 10 cc. ,, = II
,, ,, 1 % + 20 cc. ,, = III
,, ,, 0·354 % + 5 cc. ,, = IV
,, ,, 0·1 % + 10 cc. ,, = V
100 cc. ,, 2 % + 100 cc. ,, = VI

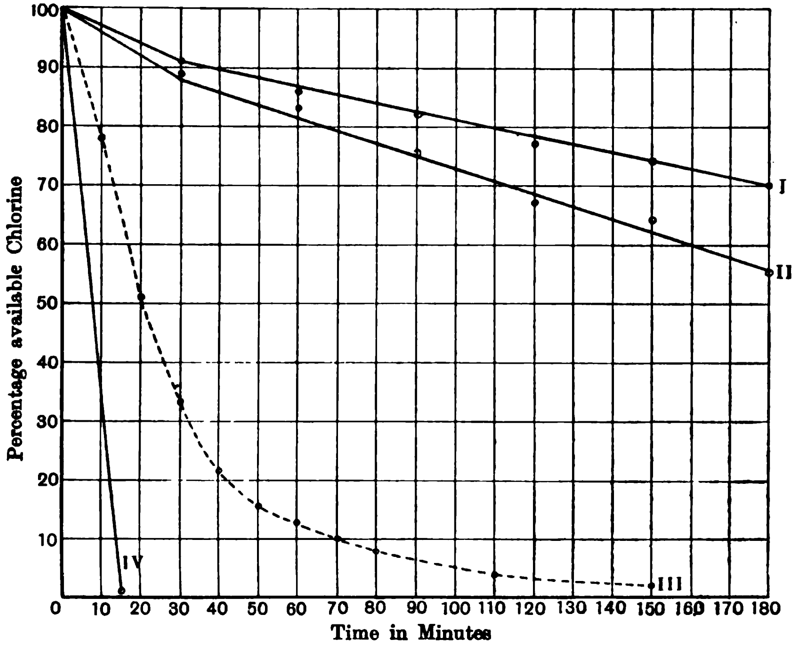
Fig. 4.

The fall in "available" chlorine appears to be a linear one of slow velocity usually with a short initial period with a more rapid rate of change. It is evident that with albumin concentrations such as were tested with hypochlorites the rate of fall in available chlorine is much slower in the case of chloramine and varies directly with the serum concentration. It is in the later stage of the hypochlorite-albumin reaction that one obtains a rate of change similar to that observed with chloramine. Even with chloramine-albumin mixtures, especially in the case of the higher albumin concentrations there is also however an initial more rapid rate of change but there is not the same tendency for the chlorine to pass into the cyclic rings in the protein, as is evident from a comparison with the hypochlorite-albumin curves. It is interesting to note that with 50 % serum in a 1 % chloramine mixture, there is a rapid initial fall of a little over 80 % chlorine during the first half hour followed by a slower rate of loss, so that at the close of 2 hours the active chlorine is about 5 % of the initial concentration. Dakin, Cohen, Daufresne and Kenyon [1916] used 50 % serum concentrations for their bacteriological tests, and as they used 1–1000 to 1–2000 chloramine concentrations the available chlorine must have been brought down to an extremely low level shortly after admixture with the serum.

THE ACTION OF HYPOCHLORITES AND CHLORAMINE-T ON AMINO-ACIDS.

Langheld [1909] has studied the action of NaOCl on various amino-acids, glycine, alanine, valine, leucine etc. He found that the α-amino-acids readily react with the hypochlorite in the same way as simple amines, forming in the first place chloramino-derivatives of the acids which, on warming, readily break down forming aldehydes or ketones, ammonia, carbonic acid and sodium chloride. The products obtained from the decomposition of glycine are formaldehyde, ammonia and carbonic acid. This change takes place very rapidly. Thus when equimolecular solutions of $Mg(OCl)_2$ and glycine (1·151 g. of the former and 0·68 g. of the latter in 200 cc.) are kept at 36°, within 15 minutes over 99 % of the "available" chlorine is lost (Fig. 5 IV). A similar mixture of equimolecular chloramine and glycine, $m/20$ in each case, shows a much less rapid fall in available chlorine (Fig. 5 III). Amino-acids such as hippuric acid, which have an acid group attached to the nitrogen offer much greater resistance to the action of hypochlorite, but at 36° slow decomposition occurs as is shown in Fig. 5 I and II. Fig. 5 I shows the fall in available chlorine when 0·812 g. hippuric acid (as sodium salt) is added to 200 cc. $Mg(OCl)_2$ $m/20$ and Fig. 5 II shows the fall when $m/40$ $Mg(OCl)_2$ is taken. The nitrogen value of the added hippurate was equal to that of 5 cc. serum. The type of fall in these cases is similar to that seen in chloramine-serum reactions and differs greatly from the hypochlorite-serum reaction. Thus an increase in the hippurate-hypochlorite ratio leads to a regular displacement downwards of the curve. Chloramine-T on the other hand shows no loss in its available chlorine even after prolonged warming with sodium hippurate.

Hypochlorite solutions therefore most readily attack the amino-acids when the N is not attached to an acid group, less readily proteins, and with greatest difficulty hippuric acid. The same order holds for chloramine-T solutions but in each case these solutions are much less effective than the hypochlorites. It seems most probable that the later slower fall in available chlorine occurring in protein-hypochlorite mixtures exhibits really the rate of change of chloramino-compounds formed by the action of the hypochlorite on the protein molecule.



200 cc. Mg (OCl)$_2$ *m*/20 + Hippuric Acid ·812 gm. = I
„ „ *m*/40 + „ „ „ = II
200 cc. containing *m*/20 Chloramine-T + *m*/20 Glycine = III
„ „ 1·151 gm. Mg (OCl)$_2$ + 0·68 gm. „ = IV

Fig. 5.

If the germicidal action of the chloramines be a function of the available chlorine, as is probably the case, then there may be an advantage in the employment of chloramine solutions in the treatment of infected wounds when it is desired to maintain the available chlorine concentration in the presence of albumin at a moderately high level. Thus in cases where fairly long intervals elapse between the dressing of wounds, the slower rate of change of the solutions of chloramine compared to those of hypochlorite might prove of advantage. In the primary disinfection of septic wounds, however, the greater rate at which the hypochlorite gives up its available chlorine in the attack on the protein molecule will undoubtedly result in a more efficient penetration and oxidation of necrosed material. This solvent action on necrosed tissue material is undoubtedly a valuable feature in the action of alkaline hypochlorites. The hypochlorite solutions will also act more rapidly on the products of bacterial action than the chloramine solutions of a similar strength.

INTRAVENOUS INJECTION OF HYPOCHLORITES AND CHLORAMINE-T.

At Professor Lorrain Smith's request the physiological effects produced by the intravenous injection of hypochlorite solutions were investigated. From the preceding study of the changes which hypochlorites undergo in albuminous solutions, one recognised that on injection into the blood there would be a very rapid fall in available chlorine taking place near the seat of injection, but there was always a possibility that the more resistant chloramine derivatives might enable the action to be sufficiently prolonged to render the blood germicidal. There was also the possibility that the powerful oxidising action of the hypochlorites might render innocuous certain products of bacterial action. This seemed much more likely than the former hypothesis, as in the cases, which have been described by Lorrain Smith and his co-workers [1915, 2], of benefit resulting from intravenous injection of comparatively small quantities of hypochlorite in the treatment of septic conditions in man, the available chlorine concentration of the blood must have been much below the germicidal level.

Hypochlorite solutions however attack the red blood corpuscles and this action must be borne in mind. Solutions of extremely low hypochlorite concentration injected in isotonic saline solution give rise to haemolysis and the pigment so set free is with greater concentrations broken down finally to colourless derivatives. The following table shows the concentrations at which haemolysis takes place when a 4 % blood corpuscle emulsion is mixed with isotonic hypochlorite and chloramine-T solutions. The tubes were left overnight at 37°.

	"Available" chlorine concentrations		
	1–20,000	1–7000	1–2500
Eusol	Very slight haemolysis	Reddish brown opaque solution	Reddish brown
Dakin's solution	„ „	Clear deep red solution	Pigment bleached yellow
Mg $(OCl)_2$ ...	„ „	„ „ „	Clear deep red solution
Chloramine-T*	No haemolysis	No haemolysis	Very slight haemolysis

* (Concentrations in terms of chloramine-T not chlorine.)

On injection into the blood laking does not occur quite so readily as in the test tube experiments, but it was met with in every case of hypochlorite injection where the available chlorine concentration of the blood had been raised to 1–3000. Probably it occurs with much lower concentrations. In practically no case did chloramine injections give rise to laking. Great care requires to be taken in the case of hypochlorite injections to prevent prolonged contact of the solution with the vessel wall as there is a gradual disintegration of the lining endothelium with a possible subsequent leakage and irritation at the seat of injection. Hence as soon as the injection is finished saline ought to be used to wash away the irritant. It is advisable to use therefore a vaselined double-way cannula, so that the saline may be injected without delay after the hypochlorite.

The intravenous injections were carried out with a view to determine the point to which the available chlorine concentration of the blood might be raised without seriously affecting the circulation and respiration. The actions of eusol, Dakin's solution, Mg $(OCl)_2$ solution and chloramine-T were investigated. As the results are much the same in the case of the various hypochlorites a short general summary will be sufficient.

The experiments were carried out on rabbits, cats and in one case on the dog. When concentrations are specified, they refer to cats of approximately 2½ kgm., unless otherwise stated.

In all cases recovery is complete when the available chlorine concentration of the blood is not raised much above 1–2000, but distinctly higher concentrations may be tolerated, for example 1–1000 or rather more. NaOCl and Mg $(OCl)_2$ may be used in slightly higher concentrations than eusol.

The first injections ought to be weaker than the later ones as the primary effects on circulation are greater than those obtained from the subsequent injections. Hypochlorite solutions containing 0·25 % "available" chlorine produce practically no disturbance until the chlorine concentration of the blood is raised to 1–1500 that is until about 40 cc. have been injected in an animal of 3 kgm. 10 cc. of a 0·5 % solution usually give rise to an initial fall of blood pressure which is quickly recovered from and the second 10 cc. is usually without effect, but on raising the concentration to a higher level the blood pressure begins to fall.

The respirations are quickened after each injection and at least in the case of $Mg(OCl)_2$ are diminished in amplitude. Eusol in concentrations of 0·35 % or higher usually gives rise immediately after injection to slightly irregular respiratory movements. Even when hypochlorite has been added in sufficient quantity to raise the concentration in the blood to 1–600 no hypochlorite or active chlorine reaction can be obtained in a sample of blood withdrawn about half an hour after the injection. If adrenaline however be injected very shortly after hypochlorite is given, care being taken to remove the latter from the seat of injection, the usual rise in blood pressure is not obtained.

This may be due to the laked blood destroying the adrenaline, as is said to occur, or may be due to destruction by oxidation.

In all cases where the chlorine concentration is 1–2000 or more, the blood plasma after centrifugalisation is deep red in tint and the spectrum is one of oxyhaemoglobin. The coagulability of the blood is decreased. The secretion of urine owing to the saline injections is increased and in most cases it is also red in colour due to the presence of free oxyhaemoglobin.

CHLORAMINE-T INJECTIONS.

The blood pressure is more readily affected than by hypochlorite injections. There is always a slight fall in blood pressure after the injection of 30 cc. 0·25 % chloramine into animals of an average weight of 2½ kilogrammes. When 40 cc. 0·25 % solution are injected the fall is not recovered from. This would give a chloramine concentration in the blood of 1–1250. Injections of 10–20 cc. 0·5 % chloramine and over give rise at first to a fairly rapid then a gradual fall in blood pressure which is usually not recovered from. At the same time respirations are slowed and there may be brief apnoeic periods. It is advisable therefore not to inject solutions of greater strength than 0·2 % and the chloramine concentration in the blood ought not to be raised above 1–2000. No haemolysis results from the chloramine injection of this concentration. Adrenaline injections give the usual rise of blood pressure even when given immediately after the chloramine. The coagulability of the blood is apparently not affected. Specimens of blood withdrawn after chloramine injections do not give the reactions of active chlorine.

Dakin and his collaborators have shown that in the presence of 50 % serum the germicidal concentration of chloramine-T is approximately 1–2000. Under the conditions of possible intravenous injection in man when comparatively small quantities of chloramine would come into immediate contact with substances which would rapidly reduce the available chlorine, it is extremely unlikely that anything approaching even a temporary germicidal concentration could be attained without producing dangerous circulatory and respiratory disturbances. Intravenous injections of hypochlorites and allied substances may however prove of value in general septic conditions in some other way than by simply rendering the blood germicidal. The extremely powerful and rapid action of the hypochlorites on the proteins and most of their amino-derivatives makes it probable that a similar action will take place on the products of bacterial action.

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XXXVII. FEEDING EXPERIMENTS WITH KYNURENIC ACID.

By CHUAI ASAYAMA.

From the Biochemical Laboratory, Cambridge.

(*Received August 29th, 1916.*)

In the endeavour to bring to light intermediate stages in the metabolic breakdown of individual amino-acids the method of feeding an animal with substances which, on chemical grounds, represent possible intermediate products has been widely employed. If a substance so administered can, under suitable circumstances, be shown to yield a characteristic end product common to it and to the original amino-acid its claim to be a normal intermediary becomes fairly well established. The light which was thrown upon the metabolism of tyrosine and phenylalanine when Otto Neubauer administered hydroxyphenyl-lactic acid, and hydroxyphenylpyruvic acid to alcaptonuric individuals is a familiar case in point.

A product appearing in the intermediate processes of metabolism is not necessarily a degradation product in the narrower sense of the term. It may be something other than a link in the chain of processes which yield energy alone. The substance may itself subserve some function in the body, or it may be a stage in the production of a substance with a function. If an amino-acid from protein were the normal precursor of a metabolite possessed of some particular functional significance, one of the effects of withdrawing that amino-acid from the food would clearly be an interference with the function concerned. In such a case it seems probable that in the absence of the amino-acid itself the animal would benefit if an intermediate product were administered. On these lines the metabolic importance of a substance known to arise from a given amino-acid may be tested by giving it in the food in place of the parent substance. Kynurenic acid, for instance, may be fed in place of tryptophane.

A supply of tryptophane is known to be essential to the animal, which seems unable to synthesise it. While it is of course required for the formation of tissue proteins, and therefore for growth, it is probable that it also yields products with more specialised functions [Hopkins and Willcock, 1906]. Is kynurenic acid such a product, or the precursor of such a product?

Since Ellinger showed that kynurenic acid arises from tryptophane the precise significance of the appearance of a quinoline derivative during the metabolism of the indole nucleus has become a matter of interest.

Kynurenic acid might be a product on the main line of the destructive breakdown of tryptophane; it might be the end product of some accessory, non-significant, process; it might be, on the other hand, the precursor of a substance with a special function.

Miss A. Homer [1915] believes that the first possibility should be rejected, as her experiments show that (in the dog) kynurenic acid given subcutaneously, or by the mouth, is almost quantitatively excreted in the urine, though when tryptophane itself is given the amount of kynurenic acid excreted is only a small fraction of what would correspond to the metabolised tryptophane. According to Miss Homer a certain amount of the latter is side-tracked into the form of the quinoline derivative, while the greater part is broken down on other lines, or converted into substances of significance to the body.

But the fact that an intermediate metabolite is excreted more or less quantitatively when administered in excess to an animal in a state of full nutrition does not disprove the possibility that it normally arises on a significant line of change. So long as sufficient is already being produced to subserve any supposed function the excess administered may well be excreted unchanged. Only when a gap, so to speak, is first produced in metabolism by wholly withdrawing the primary source of the substance can the effect of supplying the substance itself be properly observed.

In the experiments to be described in this paper, rats were fed upon a basal dietary containing all the amino-acids of protein except tryptophane. One set of animals was fed upon this dietary alone. To the food of a second set tryptophane was added, while a third set received kynurenic acid instead of tryptophane. The results seem to show that, in the case of the rat at any rate, kynurenic acid can in no sense replace tryptophane. It does not restore to any degree the power of maintenance which a dietary always loses when deprived of tryptophane. It produces no apparent betterment in the general health of the animal, and does not lengthen the survival period.

METHODS USED.

The basal dietary used in the experiments had the following composition:

Amino-acid mixture obtained from prolonged acid hydrolysis of caseinogen	250 g.
Potato starch	418 ,,
Cane sugar	207 ,,
Lard	125 ,,
Ash of oats	13·5 ,,
Ash of dog biscuit...	13·5 ,,

The fact that acid hydrolysis entirely destroys tryptophane without otherwise seriously interfering with the nutritive power of the mixture, makes the amino-acids from such hydrolysis a convenient source of tryptophane-free food. To prepare the dietary containing tryptophane 3·75 g. of the indole acid were added to the above mixture, while in the preparation of the third dietary 3·0 g. of kynurenic acid were added. Complete conversion of 3·75 g. tryptophane would yield 3·474 g. kynurenic acid. The latter was prepared from the urine of two dogs which had been given tryptophane by the mouth. It was carefully purified and melted at 89°. The necessary vitamine supply was given to the rats in the form of an alcoholic extract of the solids of fresh milk.

The animals were kept in a room of uniform temperature. They were fed and weighed at uniform intervals, and all three sets received precisely similar treatment.

DISCUSSION OF RESULTS.

The figures give the weight curves of the rats under experiment. The first three show those of individuals, and the fourth the average weights in the case of each experimental set. The animals were fed upon bread and milk up to the 28th day (marked by an arrow) and were then put upon the experimental dietaries.

Fig. 1 shows the characteristic loss of weight which immediately follows when tryptophane is absent, the animals of this set being upon the basal dietary without any addition.

Fig. 2 shows the effect of adding tryptophane to the basal dietary. Instead of loss of weight there is maintenance, and, as seen in the average

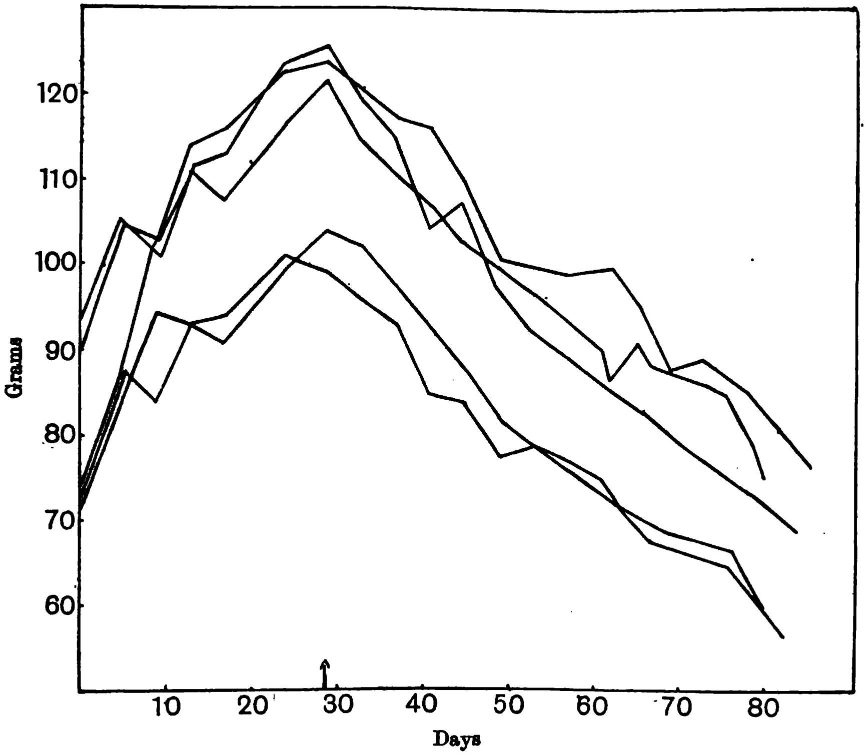

Fig. 1. Body weight curves of individual rats which from the 28th day received the basal diet alone.

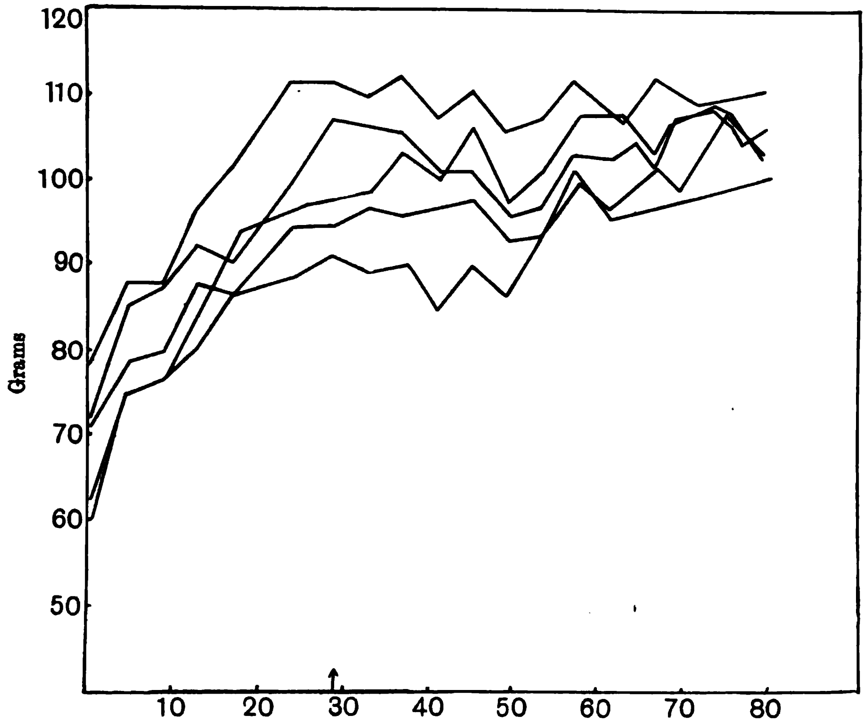

Fig. 2. Individual weight curves of rats receiving tryptophane in addition to the basal dietary.

weight curve of Fig. 4, a certain amount of growth. In a large number of experiments carried out in this laboratory, in other connections, quite normal growth has been obtained when the sole nitrogen supply was the amino-acid mixture from acid hydrolysis with added tryptophane, though a small addition of cystine has sometimes proved necessary. The somewhat less favourable result seen in my experiments was due, I think, to a deficiency in cystine. The protein used was caseinogen; the acid hydrolysis was prolonged (40 hours) and no cystine was added.

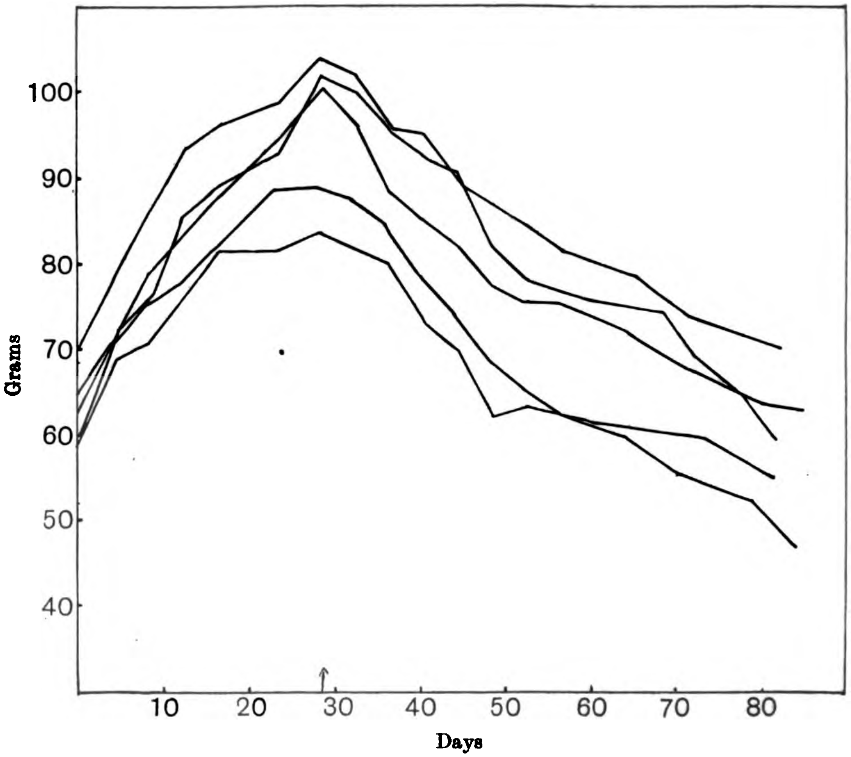

Fig. 3. Individual weight curves when kynurenic acid was added instead of tryptophane.

The effect of adding tryptophane to the basal dietary was striking enough however as will be seen from the curves. On the other hand the set of rats which received kynurenic acid instead of tryptophane showed in respect of preservation of weight no difference whatever from those on the basal dietary alone. The latter were somewhat heavier animals than the former, but it will be seen that the curves run parallel. Loss of weight occurred with equal rapidity in both. The general condition in these two sets was also strikingly similar from week to week, and the survival period was identical, being on the average exactly fifty days in each set. In sharp contrast was the condition of the set receiving tryptophane. These rats appeared normal throughout, and were in perfect health at the end of the experiment.

The consumption of food was similar in each set and was quite satisfactory throughout. The experiments show that tryptophane cannot in any sense be replaced in the food by its quinoline metabolite. It was perhaps not to be expected that it could replace it as a factor in growth, as the reactions by which it is formed are not likely to be easily reversible; but, in the rat at any rate, it would appear to be without significance to the body[1].

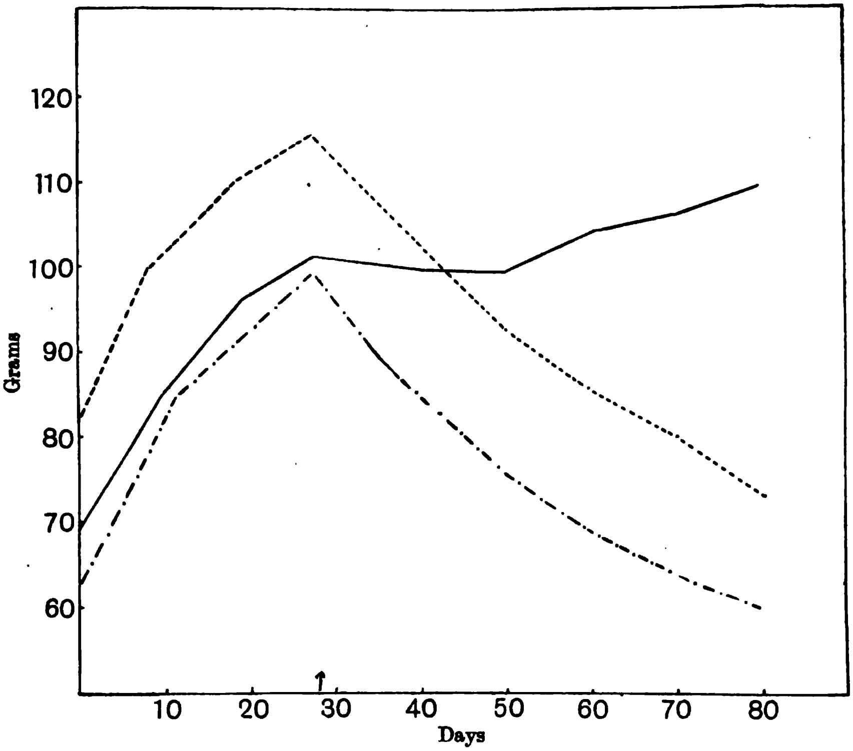

Fig. 4. Average weight curves. Dotted line, rats upon basal dietary alone. Continuous line, set upon the basal diet plus tryptophane. Dot and dash line, set taking kynurenic acid.

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[1] *Note by F. G. Hopkins.* Dr Asayama's results gain in significance if it be shown that kynurenic acid is a normal metabolite of the rat. The animal certainly excretes extremely little of the substance, but after feeding with tryptophane I have been able to separate small amounts of a substance from rats' urine which gives the reactions of kynurenic acid and melts at 88°–89°.

I have to thank Professor Hopkins for suggesting the experiments described, and for the interest he has taken in them. The administration of the dietaries was carried out under his direct supervision, and he has been responsible for the care of the animals.

SUMMARY.

When tryptophane is absent from the food of rats no betterment of nutrition is observed if kynurenic acid be given. The quinoline derivative of tryptophane seems to possess no special significance in metabolism.

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XXXVIII. A STUDY OF THE DISTRIBUTION OF NITROGEN IN THE PROTEINS OF TUMOURS AND NORMAL TISSUES.

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(*Received August 15th, 1916.*)

INTRODUCTORY.

A review of the literature dealing with the chemical aspects of the cancer problem will demonstrate how little serious attention, relatively speaking, this important subject has received at the hands of the biochemist.

The scattered facts which constitute our present knowledge of the subject have been ably collated by Wells [1914] in his text-book of Chemical Pathology.

Sufficient may be gathered from a perusal of his review to show that the chemist has been unable to demonstrate satisfactorily that the cancer cell is in any way strikingly different from the cell of the corresponding normal organ.

The proteins which enter into the composition of tumours have been investigated by several workers. Bang [1903] studied in particular the nucleoproteins of tumours, and was able to show that the nucleoprotein which he isolated from a lymphosarcoma was in all probability identical with the normal and characteristic nucleoprotein of lymph-glands.

Bergell and Dörpinghaus [1905] made a study of the amino-acids present in tumours, but they were unfortunately obliged to use for this investigation a somewhat mixed material from various sources. Upon analysis, using the "ester-method" of Fischer, they found that the amino-acids alanine, glutaminic acid, aspartic acid and phenylalanine were apparently present in a higher proportion than is usually found in normal tissues; while the yield of leucine, glycine and tyrosine was somewhat lower.

The analyses of Neuberg [1906], however, gave results which led him to express the view that the amounts of the amino-acids leucine, glutaminic acid, tyrosine and glycine found in tumour proteins were very much of the same order as those found in the normal organs.

Bergell [1907] investigated the proteins of human carcinomata and also of mouse cancers. Using the quantitative nitrogen-distribution method of Hausmann [1900] he obtained much higher values for the diamino-acid fraction in all cases of cancer than in normal. Thus, in human carcinoma he found that 40 % of the total nitrogen was present in the form of diamino-acid nitrogen, the figure for normal tissues being about 29 %. He also obtained similar results in the analysis of the mouse tumour proteins, and concluded that the diamino-acids played an important *rôle* in the cancer cell.

Blumenthal [1907] after having reviewed all the previous work, and also that of Wolff [1905], who found a relatively greater proportion of albumin than globulin in the press-juices of several tumours, as compared with normal tissues, concluded that important differences exist in the chemical nature of normal and malignant cells. Abderhalden and Medigreceanu [1910] examined a series of primary tumours of the liver in cows, several mouse carcinomas, and a sarcoma, quantitatively for the three amino-acids tyrosine, glycine, and glutaminic acid. Their figures show good agreement and indicate that the proportion of these substances present in tumours is not abnormal.

Wells [1914] also inclines to the view that the proteins of tumours are probably identical with, or at any rate closely similar to, those of normal organs. He explains the results of Bergell, to which allusion has been made, on the grounds that the majority of cancerous tissues are very cellular, and are therefore rich in nuclear material, and he quotes in support of this opinion the work of Petry [1899], who found that a carcinoma of the mammary gland contained a much larger proportion of nucleoprotein than did the normal gland.

We can now see that the majority of the work that has been reviewed agrees in regarding the proteins that enter into the composition of malignant growths as very similar in composition to those present in the normal organs, although we cannot consider many of the results as satisfactory from the point of view of quantitative analysis.

Recently a paper appeared by Kocher [1915], in which this author claims to have demonstrated that the amounts of the hexone bases, arginine, histidine, and lysine present in tumour proteins are approximately double those found in normal tissues. He used the method of analysis introduced by Kossel and Kütscher [1901], with the subsequent modifications recommended by

Osborne, Leavenworth and Brautlecht [1909], Steudel [1903], and van Slyke [1914]. One cannot but regard his analytical data as being somewhat insufficient, for he has only submitted five tumour proteins to analysis, and has then compared the results of these analyses with figures published some time previously by Wakeman [1908] for the diamino-acid content of normal organs. The actual tumours examined by Kocher were of a somewhat miscellaneaus nature, namely, a metastatic deposit in the liver secondary to a primary carcinoma of the stomach, a lymphosarcoma, an endothelioma, a primary carcinoma of the larynx and another secondary deposit in the liver from a primary carcinoma of the ileum.

Shortly before the appearance of Kocher's paper an examination along very similar lines was begun in this Institute as part of a detailed investigation into the chemical consideration of the cancer problem.

This examination consisted of a study of the distribution of nitrogen in the proteins of tumours, as compared with normal tissues, by the method of van Slyke.

One particular type of tumour was chosen at the outset for more or less exhaustive study, as it was realised that in order to be justified in drawing conclusions from the results obtained, it was necessary to compare examples of variations of one type of tumour—for instance the slow and rapidly growing forms—with one another, as well as with the normal organ.

The actual growth selected for study was cancer of the breast, and the reasons for this choice were several. Firstly, it is possible to obtain tumours of this variety with comparative ease, secondly, there are a large number of forms of cancer of the breast which vary in their rate of growth considerably, while for a third reason, it is possible to obtain specimens of pathological breast tissue in a condition which is regarded by many as being one of the so-called "pre-cancerous" states. On the publication of the summary of Kocher, however, the study was extended to an experimental neoplasm of the chicken which is receiving considerable attention in this Institute, namely, the Rous sarcoma.

From the summary of the paper by Kocher one is led to assume that this author regards the high values for the hexone bases in tumours as being in some way responsible for the abnormal rate of growth of these tissues, particularly in the case of the diamino-acid lysine.

It was therefore felt that this point could only be decided by an investigation of a tumour that could be controlled experimentally, and accordingly the neoplasm referred to above was chosen.

It is necessary to say that although many points remain to be decided before we can classify this particular tumour as a sarcoma, yet, for many reasons it was felt that its characters sufficiently resemble a malignant growth to justify such a study.

Indeed it would be out of place to discuss here the evidence on either side of the question as to whether the growth is one of the group of diseases known as the infective granulomata, or whether it is a form of cancer, for it is hoped that a certain amount of light on that point will be shed by the researches which are being made in this Institute at the present time.

By carefully adjusting the conditions under which this growth is propagated it is possible to vary to a considerable extent its virulence and its rapidity of growth. Originally the tumour was discovered by Rous [1910] in a hen of the Plymouth Rock strain, and for some time it was only possible to carry on the tumour by inoculating grafts when the host was a pure bred chicken of that particular breed; other strains apparently possessed a natural form of immunity. Passage of the tumour through a very large number of generations has, however, had the effect of decreasing the immunity enjoyed by the other breeds to some extent, so that if injected into a fowl of the Minorca or White Leghorn variety growth of the graft will sometimes be obtained, although usually its rate will be slow. The rate of growth in its natural host, the Plymouth Rock chicken, is generally very rapid, although even in these cases the rate is greatly influenced by the age and state of health of the host.

By a selection of suitable conditions it is therefore possible to control to some extent the rate of growth of this neoplasm; it being borne in mind that in all probability many of the conditions applied are not artificial or unnatural ones, but ones that in many cases do bear an important part in influencing the growth of human cancer. There is, unfortunately, one drawback to the use of this particular tumour for such a study as was intended, that being that it is a growth which simulates a true sarcoma in being a proliferative condition of the connective tissue of the fowl; it is therefore difficult to obtain a normal chicken tissue with which to make an adequate comparison. This point was, however, duly taken into consideration, together with the arguments in favour of using the growth, with the result that it was decided that the results of the analyses of the human tumours would serve as a control to some extent in this respect.

EXPERIMENTAL.

Methods of Analysis.

The proteins were in every case examined by the nitrogen distribution method of van Slyke [1911], utilising the modifications in the treatment of the phosphotungstate precipitate introduced by the same author at a later date [1915]. On arrival in the laboratory the tissues were immediately freed from all foreign tissue and finely minced in a small hand-mincing machine. The proteins of the minced tissue were then coagulated by boiling, a small quantity of dilute acetic acid being added to the boiling water. The coagulated protein was filtered off, washed with water containing a small trace of acetic acid, pressed and dried. Where necessary, traces of residual protein in solution in the filtrate were recovered by precipitation with alcohol, and added to the main portion.

The dried protein was then rendered fat-free by continuous extraction in a Soxhlet extractor with ether, after which the fat-free product was ground to a fine state of division and passed through a fine mesh sieve.

In most cases approximately 3 g. of the resulting greyish white powder was hydrolysed with 30 cc. of 20 % hydrochloric acid until hydrolysis was complete, as shown by determinations of the amino-nitrogen, the treatment of the resulting solution being then carried out along lines almost identical with those devised by van Slyke [1911].

One difficulty encountered was in the estimation of arginine, for it was found that to obtain reliable duplicate titrations was a matter of great uncertainty when the process of van Slyke was used. Plimmer [1916] has also had occasion to draw attention to this fact, and has suggested several slight modifications which undoubtedly improve the accuracy of the process.

Throughout the course of this research it has been the practice to estimate the total nitrogen of the diamino-acids fraction in a separate portion of the solution of the bases, and not in the residual liquid remaining after the determination of the arginine. Plimmer also recommends this procedure. The other improvements mentioned in his paper, namely, the use of a more dilute alkali for the hydrolysis of the arginine, and the distillation of the last traces of ammonia resulting from this hydrolysis by removing the condenser water and continuing the boiling for some twenty minutes, were adopted during the latter part of this work.

TABLE I.

Distribution of Nitrogen in the Proteins of Human Tumours.

Percentages of total nitrogen.

No. of case	Age	Weight of tumour g.	Approximate time of growth*	Characteristics of the tumour	Amide N	Humin N	Mon-amino N of filtrate	Non-amino N of filtrate	Arginine N	Histidine N	Lysine N	Cystine N	Total N of diamino-acids
331A	63	33	24 months	Scirrhus carcinoma of breast, slow growing, with hyaline degeneration of stroma and vacuolation of the protoplasm	4·42	2·24	52·20	11·80	9·45	7·50	12·39	0·70	30·04
331B	—	32	—	Rapidly growing metastatic deposits in the axillary glands from Case 331	4·72	2·47	53·30	3·75	8·40	8·10	16·44	0·96	33·90
347	63	81	6 „	Scirrhus carcinoma of breast, slow growing but glandular type, somewhat fibrous	5·35	1·72	57·80	11·90	10·21	7·98	4·26	1·55	24·00
342A	41	48	12 „	Scirrhus carcinoma of breast, slow growing with much fibrosis	3·86	2·15	50·80	12·45	9·18	6·27	14·55	—	29·90
343A	52	25	12 „	Scirrhus carcinoma of breast, slow growing	4·36	2·02	52·30	10·62	16·75	1·64	11·41	—	29·80
343B	—	42	—	Secondary deposits from glands of axilla from Case 343, actively growing	4·75	1·97	59·00	4·10	12·00	7·90	9·60	—	29·50
384A	57	56	6 „	Scirrhus carcinoma of breast, more cellular than above cases	5·87	2·84	54·90	7·40	9·51	5·77	12·72	—	28·00
384B	—	24	—	Very active metastatic deposits in glands of axilla from Case 384	3·17	2·96	59·20	4·00	9·31	7·10	14·49	—	30·90
387A	52	29	13 „	Scirrhus carcinoma of breast, fairly cellular and more active	6·10	2·22	51·80	8·20	11·85	6·29	11·91	1·15	31·20

387B	—	20	—	Rapidly growing secondary growths in glands from axilla from Case 387	5·03	2·50	52·90	3·42	13·10	8·00	13·67	1·03	35·80
18A	45	39	14 ,,	Carcinoma of breast, alveolar type, but not rapidly growing	4·90	2·02	55·55	8·72	8·15	7·10	12·55	—	27·80
18B	—	19	—	Metastatic deposits in axillary glands from Case 18	6·19	2·49	57·80	6·37	7·90	8·80	11·60	—	28·30
332	30	87	—	Cystic mastitis, much fibrosis, no evidence of malignancy	4·57	2·57	56·89	12·89	8·69	7·23	4·80	1·28	22·00
333	19	102	24 ,,	Cystic mastitis, much fibrosis, no evidence of proliferation of glandular tissue	3·01	3·09	51·10	12·43	8·91	6·44	13·55	—	28·90
349	47	84	3 weeks	General fibrosis of breast, no proliferation of glandular elements	3·74	1·39	52·70	13·70	10·75	6·86	9·20	1·20	28·01
353	49	180	3 months	General fibrosis of breast, no malignancy	3·47	1·26	51·36	11·55	13·95	6·25	10·70	1·10	32·00
20	54	60	24 ,,	Myxosarcoma of breast, slow growing	4·99	1·63	53·75	9·19	12·15	6·76	10·49	0·61	30·01
97	72	25	6 ,,	Adenocarcinoma of breast, fairly rapidly growing	5·76	2·29	58·80	3·83	10·90	7·00	9·90	1·60	29·40
103A	36	47	6–7 ,,	Encephaloid carcinoma of breast, extending rapidly	4·19	2·42	55·40	3·03	11·10	7·75	16·24	—	35·09
103B	—	39	—	Rapidly growing secondary deposits in glands from axilla from Case 103	5·62	3·32	53·10	2·85	12·30	8·70	14·20	—	35·20
110	58	600	36 ,,	Fibromyoma of uterus	5·21	1·62	52·50	10·00	9·45	7·70	12·25	—	29·40
16	20	900	6 ,,	Exceedingly rapidly growing lymphosarcoma of cervical glands	4·82	4·03	50·30	2·10	11·90	8·90	16·27	1·13	38·20

* A rough approximation of the duration of growth of the tumour derived from the observations of the patients. It must be borne in mind, however, that considerable growth may have occurred before a tumour makes its presence apparent, while there is no means of ascertaining at what stage involvement of the axillary glands occur.

TABLE II.

Distribution of Nitrogen in the Proteins of the Rous Sarcoma.

Percentages of total nitrogen.

No. of case	Weight of tumour g.	Time of growth days	Characteristics of tumour	Amide N	Humin N	Mon-amino N of filtrate	Non-amino N of filtrate	Arginine N	Histidine N	Lysine N	Cystine N	Total N of diamino-acids
5	32	30	Slow growing, somewhat fibrous	4·98	5·48	45·98	6·82	14·39	8·10	11·30	0·98	34·77
117	39	36	Somewhat fibrous small central area of mucoid degeneration	6·35	4·97	45·60	7·70	10·92	10·30	12·68	0·90	34·70
125	75	75	Very slowly growing, much fibrosis	6·25	6·25	51·30	10·10	11·94	7·59	11·05	0·82	31·40
12	32	40	Firm, slightly fibrotic tumour	4·04	5·52	43·10	8·10	11·50	11·22	13·13	0·85	36·70
3	45	42	Firm tumour, with area of central necrosis ...	6·32	6·15	48·50	7·30	11·50	9·10	10·28	0·77	31·65
90	90	30	Fairly rapidly growing tumour, with central area of mucoid degeneration	4·78	4·03	47·90	6·40	10·34	9·77	13·80	0·99	34·90
91	78	30	Firm, steadily growing tumour	5·15	1·11	48·30	6·10	11·41	10·11	12·75	1·13	35·40
133	83	21	Firm, rapidly growing, area of central necrosis	5·85	3·24	—	—	11·62	9·72	12·45	0·91	34·70
25	43	18	Fairly rapidly growing tumour, much central necrosis and mucoid change	3·98	4·95	52·40	5·70	10·72	10·00	11·90	0·78	33·40
100	70	30	Firm tumour, with some slight necrosis ...	4·68	3·66	47·70	5·60	10·44	10·12	13·48	0·66	35·70
101	68	27	Firm, solid, steadily growing tumour	6·43	3·43	48·00	5·00	10·41	10·60	13·63	1·06	35·70
72	108	18	Rapidly growing tumour, much necrosis of a myxomatous type	6·81	6·49	47·20	4·90	10·56	8·20	13·88	0·76	33·40
96	165	18	Very rapidly growing, no necrosis	6·81	6·60	50·75	4·65	11·00	10·52	10·49	0·89	32·90
139	136	36	Firm, very rapidly growing tumour	5·15	5·23	47·20	4·40	13·05	14·35	8·78	0·82	37·00
94	200	21	Rapidly growing, much central necrosis ...	6·43	5·72	50·01	4·38	12·81	8·05	10·63	0·91	32·40
94A	—	—	Metastatis in liver from 94	6·28	4·82	52·70	4·10	12·55	9·28	8·99	0·58	31·40
94B	—	—	Metastatis in lungs from 94	5·52	5·19	49·40	4·80	11·85	8·91	11·62	0·62	33·00
77	215	15	Very rapidly growing tumour, much central necrosis	5·68	4·93	46·50	4·30	11·16	12·82	12·15	0·64	36·77
60	307	51	Rapidly growing tumour, extensive central myxomatous degeneration	4·70	4·28	48·50	5·30	10·30	9·54	15·84	0·82	36·50

TABLE III.

Distribution of Nitrogen in Proteins of Normal Organs. Percentages of total nitrogen.

Organ	Remarks on organ	Amide N	Humin N	Monamino N of filtrate	Non-amino N of filtrate	Arginine N	Histidine N	Lysine N	Cystine N	Total N of diamino-acids
				I. *Human.*						
Pancreas ...	Normal	5·52	4·50	53·27	3·63	11·30	8·82	10·91	0·97	32·00
Kidney ...	,,	5·05	3·24	59·30	5·10	11·95	5·89	7·00	1·01	25·85
Spleen... ...	,,	5·18	3·82	47·62	4·88	12·50	13·50	11·00	1·10	38·10
Cardiac muscle	,,	5·67	1·55	52·95	5·90	9·38	10·20	12·50	0·82	32·90
Skeletal muscle	,,	6·18	2·29	58·90	4·41	11·05	2·72	13·60	0·80	28·17
Liver	,,	6·12	3·32	53·85	4·35	10·50	8·20	11·76	1·34	31·80
Cardiac muscle	,,	6·35	3·60	53·20	5·00	10·00	10·00	9·25	0·80	30·05
Kidney ...	,,	6·05	3·57	52·80	5·28	11·45	8·60	9·25	1·10	30·40
Skeletal muscle	,,	6·42	2·30	57·00	4·20	11·30	5·79	12·71	0·90	30·70
Pectoral muscle	Removed with breast No. 103	5·42	2·46	58·30	4·20	11·85	4·88	10·41	0·85	27·99
Breast... ...	Normal, but slightly fibrotic	5·50	2·50	57·17	6·40	10·10	4·66	12·47	—	27·23
,, ...	,, ,, ,,	5·10	2·85	56·20	6·00	10·02	2·81	13·38	—	26·21
				II. *Chicken.*						
Lungs... ...	Normal	7·02	4·54	51·20	7·40	11·25	7·22	11·11	1·32	30·90
,, ...	,,	6·58	3·82	50·70	6·70	12·20	8·42	10·98	1·40	33·00
Liver	Stunted chick[1]	7·49	4·40	56·50	3·58	9·09	8·10	11·24	1·27	29·70
,, ...	Normal	8·19	5·56	54·80	4·95	9·15	8·00	9·25	1·30	27·70
,, ...	,,	8·17	5·52	55·20	4·00	9·45	7·44	9·45	1·16	27·50
Pectoral muscle	Stunted chick[1]	6·08	2·20	57·90	4·75	10·88	5·24	10·82	0·86	27·80
,, ,,	Normal	6·55	1·77	57·85	4·36	11·10	9·71	8·89	0·90	30·60
,, ,,	,,	6·72	1·75	56·15	4·55	11·10	7·20	10·74	0·91	29·95
Cardiac muscle	Stunted chick[1]	6·47	3·42	59·50	5·08	8·60	7·40	9·37	0·88	26·25
,, ,,	Normal	6·64	3·34	54·00	5·80	11·40	7·72	7·63	0·85	27·60
,, ,,	,,	6·02	3·17	58·05	5·10	9·99	8·00	8·36	0·90	27·25
Brain	,,	10·57	5·25	67·95		7·35	2·72	5·03	0·90	16·10

[1] Undersized chicks exhibiting signs of malnutrition through being reared from an early age indoors.

TABLE IV. *Analytical Data for*

							Monamino-acid fraction vol. 500 cc.			
No. of case	Total N vol. 100 cc. N in 5 cc. cc. 0·1 N acid	Total N	Amide N vol. 90 cc. cc. 0·1 N acid	Amide N	Humin N vol. 90 cc. cc. 0·1 N acid	Humin N	N in 25 cc. cc. 0·1 N acid	Total N in filtrate	cc. N gas from 10 cc.	Amino N of filtrate
331A	15·00 15·15	0·4228	12·00	0·0187	6·20	0·0096	8·40 8·30	0·2600	6·62, 19·5° C., 762 mm.	0·2103
331B	14·80 14·90	0·4160	12·60	0·0196	6·65	0·0103	7·70 7·55	0·2370	7·09, 19° C., 750 mm.	0·2220
347	12·40 12·20	0·3440	11·80	0·0184	3·80	0·0059	7·60 7·80	0·2400	6·26, 19·5° C., 762 mm.	0·1985
342A	13·60 13·65	0·3810	8·40 (*k*)	0·0147	4·60	0·0082	6·70 6·85	0·2380	5·49, 16·5° C., 743 mm.	0·1935
343A	12·85 13·00	0·3612	10·10	0·0157	4·70	0·0073	7·30 7·20	0·2270	5·92, 17·5° C., 762 mm.	0·1890
343B	13·30 13·50	0·3750	13·30	0·0178	4·70	0·0073	7·70 7·55	0·2370	7·06, 19° C., 751 mm.	0·2210
384A	15·30 15·10	0·4258	16·10	0·0250	7·80	0·0121	8·55 8·50	0·2680	7·20, 15° C., 762 mm.	0·2340
384B	9·30 9·45	0·2630	5·30	0·0082	5·00	0·0078	5·30 5·30	0·1660	4·97, 16·5° C., 743 mm.	0·1656
387A	17·75 17·75	0·4973	19·45	0·0303	7·10	0·0110	9·75 9·45	0·2980	8·20, 16·5° C., 743 mm.	0·2570
387B	15·00 15·00	0·4200	13·60	0·0212	6·80	0·0105	7·60 7·60	0·2370	6·82, 15° C., 762 mm.	0·2220
18A	14·20 14·40	0·4004	12·60	0·0196	5·20	0·0081	8·20 8·30	0·2570	6·82, 15° C., 762 mm.	0·2220
18B	5·55 5·60	0·1568	6·25	0·0097	2·50	0·0039	3·25 3·24	0·1008	2·89, 24° C., 766 mm.	0·0906
332	14·60 14·80	0·4120	12·10	0·0188	6·80	0·0105	9·40 9·20	0·2900	7·20, 14·5° C., 762 mm.	0·2340
333	12·40 12·40	0·3470	5·20 (*m*)	0·0104	5·40	0·0107	5·30 5·70	0·2202	4·20, 13·5° C., 764 mm.	0·1770
349	16·90 16·90	0·4740	11·40	0·0176	3·90	0·0066	10·20 9·95	0·3145	7·92, 19° C., 750 mm.	0·2490
353	16·20 16·25	0·4540	10·10	0·0157	3·60	0·0057	9·10 9·25	0·2865	7·49, 16·5° C., 743 mm.	0·2340
20	11·80 11·90	0·3332	10·65	0·0166	3·40	0·0054	6·70 6·70	0·2090	5·72, 24° C., 766 mm.	0·1785
97	8·80 8·95	0·2492	9·20	0·0143	3·60	0·0057	5·00 5·00	0·1560	4·66, 24° C., 766 mm.	0·1460
103A	16·35 16·05	0·4540	12·20	0·0190	7·10	0·0110	8·55 8·45	0·2650	7·91, 18·5° C., 765 mm.	0·2520
103B	16·00 15·85	0·4452	16·10	0·0250	9·50	0·0148	8·00 8·00	0·2490	7·48, 18·5° C., 765 mm.	0·2370
110	17·50 17·70	0·4930	16·40	0·0256	5·10	0·0080	9·90 9·85	0·3080	8·13, 18·5° C., 765 mm.	0·2590
16	15·80 15·60	0·4400	13·60	0·0212	11·45	0·0177	7·50 7·30	0·2310	7·05, 21·5° C., 760 mm.	0·2220

(*k*) Estimations carried out on 80 cc.
(*m*) Estimations carried out on 70 cc.

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Diamino-acid fraction vol. 50 cc.								
N in 5 cc. cc. 0·1 N acid	Total N of bases	cc. N gas from 5 cc.	Amino N of bases	Arginine N in 10 cc. cc. 0·1 N acid	Arginine N	g. BaSO$_4$ from 5 cc.	Cystine N	Total N recovered
8·30 8·20	0·1300	11·90, 15° C., 765 mm.	0·0763	2·60	0·0405	0·0009	0·0030	0·4183
9·10 9·10	0·1410	12·22, 15° C., 765 mm.	0·1018	2·30	0·0350	0·0012	0·0040	0·4079
5·40 5·20	0·0825	6·15, 15° C., 765 mm.	0·0397	2·25	0·0350	0·0016	0·0053	0·3468
6·55 6·65	0·1145	10·20, 17° C., 750 mm.	0·0729	2·00	0·0350	—	—	0·3754
6·90 6·80	0·1070	8·90, 15° C., 765 mm.	0·0581	3·90	0·0605	—	—	0·3570
7·10 7·10	0·1105	8·75, 15° C., 765 mm.	0·0570	2·90	0·0450	—	—	0·3726
7·65 7·70	0·1200	11·30, 15° C., 765 mm.	0·0725	2·60	0·0405	—	—	0·4251
5·20 5·20	0·0810	7·80, 15° C., 765 mm.	0·0510	1·60	0·0245	—	—	0·2630
9·90 9·85	0·1545	14·12, 22° C., 760 mm.	0·0889	3·80	0·0590	0·0017	0·0057	0·4828
9·65 9·70	0·1510	13·90, 22° C., 760 mm.	0·0873	3·52	0·0550	0·0013	0·0043	0·4180
7·20 7·10	0·1110	10·60, 21° C., 753 mm.	0·0669	2·10	0·0325	—	—	0·3957
2·85 2·85	0·0444	4·00, 15·5° C., 765 mm.	0·0258	0·80	0·0124	—	—	0·1587
5·70 5·85	0·0905	6·90, 17° C., 750 mm.	0·0436	2·30	0·0357	0·0016	0·0053	0·4098
4·85 5·00	0·1000	5·94, 17° C., 750 mm.	0·0509	1·55	0·0310	—	—	0·3113
8·50 8·50	0·1325	11·25, 15° C., 765 mm.	0·0724	3·35	0·0510	0·0017	0·0057	0·4712
9·30 9·20	0·1445	11·50, 15° C., 765 mm.	0·0772	4·10	0·0635	0·0015	0·0050	0·4524
6·50 6·35	0·1000	8·70, 21·5° C., 757 mm.	0·0546	2·65	0·0405	0·0006	0·0020	0·3310
4·70 4·70	0·0730	5·95, 21·5° C., 757 mm.	0·0389	2·20	0·0272	0·0012	0·0040	0·2490
10·22 10·20	0·1590	15·70, 22° C., 758 mm.	0·0972	3·25	0·0505	—	—	0·4541
10·00 10·20	0·1570	14·25, 22° C., 758 mm.	0·0894	3·55	0·0550	—	—	0·4458
9·20 9·40	0·1450	13·20, 18·5° C., 765 mm.	0·0818	2·97	0·0465	—	—	0·4866
10·70 10·85	0·1680	16·05, 21° C., 753 mm.	0·1061	3·40	0·0525	0·0015	0·0050	0·4379

TABLE V. *Analytical Data for*

							Monamino-acid fraction vol. 200 cc.			
No. of case	Total N vol. 100 cc. N in 5 cc. cc. 0·1 N acid	Total N	Amide N vol. 95 cc. cc. 0·1 N acid	Amide N	Humin N vol. 95 cc. cc. 0·1 N acid	Humin N	N in 25 cc. cc. 0·1 N acid	Total N in filtrate	cc. N gas from 10 cc.	Amino N of filtrate
5	15·65	0·4385	14·60	0·0215	16·30	0·0240	19·65	0·2320	17·00, 20°, 752	0·2020
117	16·00 (*a*)	0·4480	18·25	0·0284	14·30	0·0222	19·00	0·2360	15·70, 18·5°, 770	0·2037
125	14·90	0·4178	17·70	0·0261	17·70	0·0261	8·65 (*c*)	0·2550	8·98, 18°, 766	0·2133
12	14·30	0·4010	11·00	0·0162	15·05	0·0222	18·10	0·2140	14·60, 20°, 752	0·1730
3	14·30	0·4010	17·20	0·0254	16·80	0·0247	7·60 (*c*)	0·2240	6·35, 17·5°, 764	0·1940
90	14·50	0·4070	13·20	0·0195	11·10	0·0164	18·75	0·2210	16·10, 16°, 754	0·1950
91	15·50	0·4348	15·20	0·0224	3·25	0·0048	20·10	0·2360	17·30, 16°, 754	0·2100
133	16·90 (*b*)	0·4730	16·80	0·0277	9·30	0·0153	—	—	—	—
25	16·05 (*a*)	0·4495	11·70	0·0179	14·30	0·0223	17·55	0·2365	18·85, 18·5°, 770	0·2355
100	13·60	0·3820	12·10	0·0179	9·50	0·0140	17·35	0·2040	15·20, 18°, 750	0·1819
101	14·40	0·4040	17·50	0·0258	9·30	0·0137	18·10	0·2140	16·20, 18°, 750	0·1935
72	14·50	0·4070	18·60	0·0274	17·90	0·0264	7·20 (*c*)	0·2120	6·28, 17·5°, 764	0·1920
96	14·80	0·4150	19·20	0·0283	16·80	0·0274	7·80 (*c*)	0·2300	6·88, 17·5°, 764	0·2100
139	14·80	0·4150	14·50	0·0214	14·70	0·0217	18·10	0·2140	16·50, 20°, 752	0·1960
94	15·80	0·4425	19·30	0·0285	17·10	0·0253	8·20 (*c*)	0·2410	7·45, 19·5°, 757	0·2220
94A	15·47	0·4335	18·40	0·0272	14·10	0·0208	8·35 (*c*)	0·2460	7·68, 20°, 757	0·2290
94B	14·40	0·4040	15·10	0·0223	14·20	0·0209	7·40 (*c*)	0·2180	6·65, 18°, 754	0·1990
77	16·90 (*a*)	0·4730	17·20	0·0268	15·00	0·0233	19·35	0·2410	16·92, 18·5°, 771	0·2200
60	22·20	0·6210	19·80	0·0292	18·08	0·0266	28·35	0·3350	24·00, 20°, 752	0·3070

(*a*) Volume of liquid carried on for analysis 90 cc.
(*b*) Volume of liquid carried on for analysis 85 cc.
(*c*) Volume of monamino-acid fraction 500 cc.

In spite of these improvements in the technique of this particular estimation, it has been found difficult to obtain concordant results. The figures for arginine are therefore not regarded as being more than approximately accurate. There is reason to believe that the values given for this amino-acid are lower than they should be, and consequently those for histidine are somewhat high.

Corrections for the solubilities of the phosphotungstates of the basic amino-acids have not been applied as results of comparative value were alone required.

The human tumour tissues utilised in this work were in all cases obtained from the Pathological Department of the Cancer Hospital. The majority of these cases were specimens removed at operation, and were in all cases available for analysis from 6 to 12 hours subsequently. One or two of the tissues were obtained post-mortem, and in these cases the time elapsing between death and the treatment of the tumour tissue was usually somewhat longer.

Proteins of Rous Sarcoma.

Diamino-acid fraction vol. 50 cc.									
N in 5 cc. cc. 0·1 N acid	Total N of bases	cc. N gas from 5 cc.	Amino N of bases	Arginine N in 10 cc., cc. 0·1 N acid	Arginine N	g. $BaSO_4$ from 5 cc.	Cystine N	Total N recovered
10·30	0·1520	13·50, 18·6° C., 752 mm.	0·0805	4·30	0·0632	0·0015	0·0043	0·4295
10·00	0·1558	13·65, 18·5° C., 771 mm.	0·0883	3·15	0·0490	0·0015	0·0040	0·4424
8·90	0·1310	12·00, 18° C., 750 mm.	0·0715	3·40	0·0500	0·0012	0·0034	0·4382
10·05	0·1475	12·10, 18·5° C., 756 mm.	0·0727	3·30	0·0462	0·0012	0·0034	0·3999
9·60	0·1290	11·60, 19° C., 761 mm.	0·0700	3·30	0·0462	0·0011	0·0031	0·4031
9·65	0·1415	13·85, 19° C., 761 mm.	0·0837	2·85	0·0420	0·0014	0·0040	0·3984
10·40	0·1535	14·39, 19° C., 761 mm.	0·0869	3·37	0·0496	0·0017	0·0048	0·4167
9·95	0·1640	13·45, 17·5° C., 766 mm.	0·0919	3·35	0·0591	0·0017	0·0043	—
9·60	0·1490	12·80, 18·5° C., 772 mm.	0·0829	3·10	0·0483	0·0013	0·0035	0·4257
9·28	0·1632	13·10, 18·5° C., 772 mm.	0·0804	2·72	0·0400	0·0009	0·0025	0·3991
9·78	0·1440	13·63, 18·5° C., 772 mm.	0·0837	2·86	0·0422	0·0015	0·0043	0·3975
9·20	0·1356	13·45, 19·5° C., 760 mm.	0·0810	2·92	0·0430	0·0011	0·0031	0·4014
9·30	0·1376	12·40, 19·5° C., 760 mm.	0·0745	3·10	0·0456	0·0013	0·0037	0·4233
10·40	0·1535	12·30, 19·5° C., 752 mm.	0·0732	3·67	0·0541	0·0012	0·0034	0·4106
9·70	0·1435	13·10, 19° C., 745 mm.	0·0772	3·85	0·0568	0·0014	0·0040	0·4383
9·20	0·1355	11·50, 19° C., 745 mm.	0·0678	3·70	0·0545	0·0009	0·0025	0·4295
9·00	0·1330	12·38, 19° C., 745 mm.	0·0730	3·25	0·0479	0·0009	0·0025	0·3942
11·18	0·1740	14·55, 18·5° C., 767 mm.	0·0938	3·40	0·0528	0·0011	0·0030	0·4651
15·40	0·2270	23·40, 19·5° C., 752 mm.	0·1392	4·35	0·0641	0·0018	0·0051	0·6178

It was found somewhat difficult to obtain accurate information as to the length of time during which these tumours had been growing—a point of some importance in this investigation—and it has therefore been necessary to rely on the approximate data furnished by the patients themselves. In this respect I must state my indebtedness to the Medical Staff of the Cancer Hospital for their kindness in placing at my disposal the clinical records of the cases examined. For all but three of the normal human tissues I must express my gratitude to Dr E. H. Kettle, Assistant Pathologist to St Mary's Hospital, London. They were obtained from normal subjects who had been the victims of fatal accidents.

The Rous tumours were obtained by experimental inoculation of small grafts of the tumour made in the pectoral muscle of a series of fowls. The subsequent development of the graft was then closely watched and all the particulars of its growth carefully noted. By this means it was possible to gain possession of much valuable data with regard to the growth of each individual tumour which it was seldom possible to get in the human cases.

The full particulars of the cases are not reproduced in entirety, but the points of actual importance with regard to each tumour are given in the tables so that they are in the best position for purposes of comparison.

The normal chicken organs were taken from healthy adult hens of the Plymouth Rock strain. Included in the tables of the analyses of these normal chicken organs will be seen several which are described as being taken from stunted chicks; these chicks were ones which had been reared from an early age in this Institute and which had become stunted before reaching the adult age [Drummond, 1916].

DISCUSSION OF RESULTS.

From a consideration of the analytical data recorded in the tables (I–VI), it is quite apparent that this research has entirely failed to corroborate the conclusions of Kocher. That this is so will be more easily realised if the following table is surveyed. It contains the average figures for each group of analyses made in this study compared with the averages given by him, from which he draws his conclusions.

TABLE VII.

Average Values for Distribution of Nitrogen in Proteins of Normal and Cancer Tissues.

	Amide N	Humin N	Mon-amino N of filtrate	Non-amino N of filtrate	Arginine N	Histidine N	Lysine N	Cystine N	Total N of diamino-acids
Human tumours ...	4·73	2·42	54·24	7·90	10·09	7·09	12·04	1·12	30·54
Normal human tissues	5·82	3·09	55·88	4·94	11·13	7·36	9·83	0·93	30·71
Chicken tumour ...	5·58	4·84	48·44	5·87	11·50	9·96	12·04	0·83	34·38
Normal chicken organs	7·20	3·73	55·62	5·11	10·13	7·26	9·40	1·05	27·70
Normal organs (*a*) ...	—	—	—	—	6·07	1·78	5·68	—	13·52
Human tumours (*b*)	—	—	—	—	12·42	4·86	11·23	—	28·48

(*a*) consists of the averages of a series of analyses of normal tissues made by Wakeman [1908], and quoted by Kocher [1915], for purposes of comparison with the results of the analyses of five tumour proteins made by the latter author, which are averaged at (*b*).

The normal tissues which are included in the average (*a*), however, include widely diverse organs taken from man, dog, ox, and horse.

It is also at once apparent that the great error made by Kocher was in not carrying out control estimations upon normal human tissues himself, for his results for the partition of nitrogen in the tumour proteins are of very much the same order as those which have been obtained during the course of this research. His figures for the percentages of arginine and histidine are probably more correct than the ones given in this communication, because in several cases his determinations were controlled by the isolation of these

TAB

Analytical Data for Protein

Tissue	Total N vol. 100 cc. N in 5 cc. cc. 0·1 N acid	Total N	Amide N vol. 90 cc. cc. 0·1 N acid	Amide N	Humin N vol. 90 cc. cc. 0·1 N acid	Humin N	Monamino-acid fraction vol. 5		
							N in 25 cc. cc. 0·1 N acid	Total N in filtrate	cc. N gas from 1
Pancreas 1 ...	14·15 14·30	0·3980	14·10	0·0219	11·50	0·0179	7·30 7·30	0·2265	7·20, 20° C., 75
Kidney 1 ...	15·00 15·10	0·4220	13·60	0·0212	8·80	0·0137	8·70 8·70	0·2707	8·00 8·00 22° C., 75
Spleen 1	15·95 16·10	0·4480	14·90	0·0232	9·95	0·0155	7·50 7·60	0·2350	7·10 7·05 22° C., 75
Cardiac muscle 1	14·40 14·15	0·4000	14·70	0·0227	3·95	0·0062	7·50 7·50	0·2350	6·80 6·75 22° C., 75
Skeletal muscle 1	14·40 14·55	0·4050	16·10	0·0250	6·00	0·0093	8·25 8·30	0·2575	8·06, 20° C., 7
Cardiac muscle 2	15·00 15·00	0·4200	17·20	0·0267	9·70	0·0151	7·85 7·90	0·2445	7·10 7·15 20° C., 7
Liver 2	12·95 12·90	0·3620	14·20	0·0221	7·70	0·0120	6·80 6·75	0·2100	6·42, 20° C., 7
Kidney 2 ...	14·50 14·60	0·4075	15·80	0·0246	9·40	0·0146	7·60 7·60	0·2360	6·75 6·75 20° C., 7
Skeletal muscle 2	17·30 17·30	0·4840	20·00	0·0312	7·10	0·0111	9·55 9·55	0·2960	9·32, 20° C., 7
Pectoral muscle 3	15·20 15·20	0·4260	14·80	0·0231	6·70	0·0104	8·65 8·50	0·2660	8·25, 20° C., 7
Breast 4	14·00 13·85	0·3900	13·80	0·0215	6·30	0·0098	8·00 8·02	0·2480	7·40, 20° C., 7
Breast 5	14·00 14·00	0·3925	12·85	0·0200	7·20	0·0112	7·85 7·90	0·2446	7·35, 20° C., 7

Normal

Lungs 3	13·80	0·3860	*18·40	0·0272	*11·90	0·0175	7·70	0·2260	6·50, 20° C., 7
Lungs 2	14·88	0·4160	18·60	0·0274	10·80	0·0159	8·10	0·2385	6·93, 20° C., 7
Liver 1	13·65	0·3820	19·50	0·0287	11·40	0·0168	7·80	0·2295	7·11, 20° C., 7
Liver 2	11·55	0·3240	18·10	0·0267	12·30	0·0181	6·60	0·1940	5·85, 20° C., 7
Liver 3	11·70	0·3270	18·25	0·0269	12·30	0·0181	6·60	0·1940	5·95, 20° C., 7
Pectoral muscle 1	10·20	0·2860	11·80	0·0174	4·27	0·0063	6·05	0·1780	5·45, 20° C.,
Pectoral muscle 2	15·42	0·4320	19·20	0·0283	4·90	0·0072	8·80	0·2595	8·25, 20° C.,
Pectoral muscle 3	15·90	0·4455	20·30	0·0299	5·30	0·0078	9·20	0·2705	8·25, 20° C.,
Cardiac muscle 1	7·85	0·2195	9·60	0·0142	5·10	0·0075	4·80	0·1415	4·30, 22° C.,
Cardiac muscle 2	14·45	0·4050	18·20	0·0268	9·10	0·0134	8·20	0·2420	7·20, 20° C.,
Cardiac muscle 3	9·30	0·2605	10·70	0·0158	5·60	0·0082	5·60	0·1647	4·97, 20° C.,
Brain	9·30	0·2605	18·70	0·0276	9·30	0·0137	6·00	0·1765	—

* Volume analysed 95 cc. in all this series.

LE VI.

of Normal Human Tissues.

cc.		Diamino-acid fraction vol. 50 cc.									
c.	Amino N of filtrate	N in 5 cc. cc. 0·1 N acid	Total N of bases	cc. N gas from 5 cc.		Amino N of bases	Arginine N in 10 cc. cc. 0·1 N acid	Arginine N	g. $BaSO_4$ from 5 cc.	Cystine N	Total N re-covered
nm.	0·2125	8·15 8·20	0·1270	10·90 11·00	19·5° C., 763 mm.	0·0700	2·90	0·0450	0·0011	0·0037	0·3933
nm.	0·2500	7·00 6·95	0·1090	8·00 7·75	20° C., 764 mm.	0·0547	3·25	0·0505	0·0012	0·0040	0·4046
nm.	0·2190	11·20 11·30	0·1750	14·55 14·60	19·5°C., 763 mm.	0·0928	3·62	0·0560	0·0015	0·0049	0·4487
nm.	0·2110	8·40 8·50	0·1315	13·50 13·60	19·5° C., 763 mm.	0·0862	2·42	0·0375	0·0010	0·0033	0·3954
nm.	0·2380	7·55 7·65	0·1180	12·20 12·30	19·5° C., 763 mm.	0·0772	2·87	0·0446	0·0010	0·0033	0·4098
nm.	0·2240	8·10 8·10	0·1260	10·40 10·44	19·5° C., 763 mm.	0·0665	2·70	0·0420	0·0010	0·0033	0·4123
nm.	0·1940	7·15 7·10	0·1115	10·40 10·40	20° C., 764 mm.	0·0663	2·45	0·0380	0·0014	0·0047	0·3556
nm.	0·2145	7·95 7·95	0·1239	10·30 10·20	21° C., 764 mm.	0·0653	3·04	0·0466	0·0013	0·0044	0·3991
nm.	0·2760	9·60 9·55	0·1485	14·30 14·00	20° C., 764 mm.	0·0889	3·50	0·0545	0·0013	0·0044	0·4868
nm.	0·2490	7·95 8·00	0·1240	11·30 11·40	20° C., 764 mm.	0·0722	3·25	0·0505	0·0011	0·0037	0·4235
nm.	0·2230	6·80 6·80	0·1060	10·10 10·00	21° C., 764 mm.	0·0644	2·53	0·0394	—	—	0·3853
nm.	0·2210	6·60 6·60	0·1030	10·39 10·30	21° C., 764 mm.	0·0660	2·53	0·0394	—	—	0·3788

cken Tissues.

c.	Amino N of filtrate	N in 5 cc. cc. 0·1 N acid	Total N of bases	cc. N gas from 5 cc.		Amino N of bases	Arginine N in 10 cc. cc. 0·1 N acid	Arginine N	g. $BaSO_4$ from 5 cc.	Cystine N	Total N re-covered
m.	0·1975	8·15 8·10	0·1195	11·30 11·40	19·5° C., 762 mm.	0·0684	2·94	0·0434	0·0016	0·0051	0·3902
m.	0·2105	9·40 9·25	0·1370	12·60 12·40	19·5° C., 762 mm.	0·0758	3·43	0·0508	0·0018	0·0058	0·4188
m.	0·2160	7·80 7·60	0·1135	11·30 11·30	18° C., 742 mm.	0·0670	2·35	0·0346	0·0015	0·0049	0·3885
m.	0·1775	6·00 6·25	0·0900	8·30 8·40	19° C., 762 mm.	0·0506	2·00	0·0295	0·0013	0·0041	0·3288
m.	0·1810	6·00 6·25	0·0900	8·30 8·35	17·5° C., 772 mm.	0·0505	2·10	0·0310	0·0012	0·0038	0·3290
m.	0·1655	5·40 5·40	0·0795	7·50 7·45	17·5° C., 772 mm.	0·0461	2·11	0·0311	0·0008	0·0025	0·2812
m.	0·2500	9 55 9·55	0·1405	12·30 12·50	17·5° C., 772 mm.	0·0765	3·26	0·0479	0·0012	0·0038	0·4355
m.	0·2500	9·00 9·10	0·1335	13·80 13·80	18° C., 742 mm.	0·0834	2·60	0·0383	0·0013	0·0041	0·4417
m.	0·1302	3·90 3·90	0·0575	4·55 4·60	18° C., 742 mm.	0·0280	1·10	0·0250	0·0006	0·0019	0·2207
m.	0·2185	7·70 7·55	0·1120	9·10 9·30	19° C., 761 mm.	0·0565	3·14	0·0461	0·0011	0·0038	0·3942
m.	0·1510	4·80 4·85	0·0710	7·20 7·25	18·5° C., 762 mm.	0·0436	1·77	0·0260	0·0007	0·0022	0·2597
	—	2·82 2·85	0·0416	3·70 3·75	18·5° C., 762 mm.	0·0225	1·30	0·0191	0·0007	0·0022	0·2594

substances in the form of their picrolonates. It will be noticed that his values for arginine are somewhat higher, and those for histidine slightly lower than have been found in the cases recorded here; this, however, is explainable by the error which it is believed exists in the determinations of the first-named substance.

The averages given in the last table are only of value to indicate whether any striking difference exists between tumour tissues and normal tissues from the point of view of the present study, for they are not in many cases strictly comparable; thus, for example, it is not permissible to draw any definite conclusion from the comparison of the amino-acid partition of the proteins of a sarcoma and of normal voluntary muscle, for we are dealing with two totally different types of tissue. We must therefore draw our conclusions as far as possible from the comparison of the normal and malignant cell of the same type.

Owing to the great difficulty of obtaining many of the specimens one would like to examine, the possibilities of carrying this out are very restricted, and only in the case of one organ—the breast—has this ideal comparison been to some extent possible. Even in this case the ideal has, unfortunately, not been realised to the extent hoped for, owing to the difficulty in obtaining certain of the material, particularly the normal gland (Table VIII).

TABLE VIII.

Average Values for the Distribution of Nitrogen in the Proteins of Normal and Pathological Conditions of the Mammary Gland.

	Amide N	Humin N	Mon-amino N of filtrate	Non-amino N of filtrate	Arginine N	Histidine N	Lysine N	Cystine N	Total N of bases
Normal	5·30	2·67	56·68	6·20	10·06	3·73	12·92	—	26·72
General fibrosis ...	3·94	2·08	53·01	12·64	10·58	6·69	9·56	1·19	27·72
Slow growing scirrhus	4·49	2·03	53·30	11·69	11·42	5·84	10·65	1·10	28·46
Rapidly growing tumours	5·36	2·35	55·29	6·23	10·30	5·78	12·06	—	30·30
Secondary deposits in glands	4·91	2·62	55·88	4·08	10·50	8·10	13·33	—	32·60

Coming to a more detailed study of the various groups of analytical data we will first turn to the results obtained in an examination of the normal and pathological mammary gland.

Only two normal breasts have been examined, owing to the difficulty mentioned, and in both these cases there was present a slight degree of fibrosis.

Fibrosis of the breast when extensive may, and often does, give rise to consequences which make the removal of the gland advisable; in fact the condition is regarded by many as being one of the so-called "pre-cancerous" states. If this view is adopted one may fairly compare results obtained in the analysis of such a case with the normal breast on the one hand, and with the case of the fibrous scirrhus carcinoma of the breast on the other. Four examples of this excessive fibrosis of the breast have been investigated. The average percentage of nitrogen present in the form of diamino-acids is 27·72, a figure that is slightly higher than the corresponding value of 26·92 for the normal gland. The large amount of fibrous tissue present in the cases of so-called chronic mastitis is to some extent reflected in the abnormally high value for the proline group of amino-acids, which averages 12·64 % of the total nitrogen, as compared with the figure 6·2 % in the case of the protein of the normal gland.

A similarly high percentage for the nitrogen present in this form is also recorded in the analysis of the uterine fibromyoma, another structure very rich in fibrous tissue. In the examples of the slow growing scirrhus carcinomata of the breast, of which there are five, we have a tissue which besides showing a certain degree of proliferation of the glandular elements, also possesses a considerable increase in the amount of fibrous stroma. Both these facts are distinctly traceable in the analytical results; thus the diamino-acid nitrogen percentage of 28·13 is a higher figure than that recorded for either the normal gland or for the mastitic condition, while the comparatively high figure for the proline group of amino-acids denotes a larger amount of the proteins of fibrous tissue than is normal. Taking now the more rapidly growing tumours of the organ under examination, examples of which are the encephaloid carcinomata, the adenocarcinoma, and the myxosarcoma, we find that the percentage of the diamino-acids is somewhat larger, 30·92 % of the total nitrogen being present in this form, whilst the proportion of the non-amino-acid of the filtrate is considerably lower.

Once again both these results are in accordance with the fact that we are now dealing with the more cellular type of tumour, that is one richer in nuclear material and relatively poorer in connective tissue stroma.

As is well known, malignant tumours of the breast are particularly prone to form metastatic deposits in the lymphatic glands of the axilla, and it is probable that in some cases the rate of growth of these secondary deposits is relatively much more rapid than that of the primary growth, especially in the case of the slow growing scirrhus tumours, a result that is possibly due to what may be called the greatly improved environment of the secondary foci.

Six specimens of such secondary deposits have been analysed, and in each case it is possible to compare these analyses with those of the primary growths from which they are derived. The results are uniform in nature, while their average will show that their greater rate of growth, and therefore their greater content of cellular matter, is portrayed in both the high figure of 32·36 % for the diamino-acid nitrogen, and the low value of 4·08 % for the proline fraction.

The other tumours which have been analysed have given results of both interest and importance. Case No. 16 is particularly interesting as being an exceedingly rapidly growing lymphosarcoma of the cervical glands. Occurring in a young man of twenty, it attained a very large size and weighed over 900 g. in the unusually short space of six months. Microscopically the tumour was seen to be an extremely active one, containing a very large amount of nuclear substance, with little supporting tissue, and this observation is well borne out by the analytical data. The percentage of the total nitrogen present in the form of diamino-acids in this tumour is the highest recorded in the whole series of analyses, namely, 38·20. It is also of considerable interest to compare this analysis with the only analysis made upon a normal lymph-gland, that of the spleen, when it will be seen that in this normal organ we have an almost identical figure for this determination, namely, 38·10 %. Results such as these should have much weight in effectively disposing of the theory that in cancer tissues we have an abnormally high proportion of the diamino-acids.

Turning now to the analytical data recorded for the proteins of the Rous sarcoma and normal chicken tissues, we find that they are very similar in nature to those we have just considered. As has been already remarked, we are not strictly justified in drawing comparisons between these two types of tissues because no normal example of the connective tissue of the fowl has been examined; for it must be remembered that the neoplasm under consideration is one of connective tissue origin.

From the average figures of analysis it will be seen that there is a distinctly higher proportion of the diamino-acids present in the proteins of the tumours of this series than in the normal chicken tissues. This is in all probability due to the much more cellular character of the tumours, for microscopically the growth is a richly cellular structure possessing a very large amount of nuclear substance.

By the study of this particular neoplasm it was hoped that it would be possible to trace in the results the influence of the rate of growth upon the content of diamino-acids. From the summarised results given below, however (Table IX), it will be seen that this expectation has not been realised to the extent that was desired. Differences of a small nature do exist, and they are in every case in the direction which was expected, but it is probably due to the fact that the slowest growing tumour of this type is relatively rich in nuclear matter that they are not of a more striking order.

TABLE IX.

Influence of the Rate of Growth upon the Distribution of Nitrogen in the Proteins of the Rous Sarcoma.

Rate of growth[1]	Amide N	Humin N	Mon-amino N	Non-amino N	Arginine N	Histidine N	Lysine N	Cystine N	Total diamino N
Approx. 1 g. per day	5·57	5·67	46·89	8·00	12·05	9·26	11·68	0·86	33·83
From 2–4 g. per day	5·14	3·40	48·86	4·90	10·82	10·05	13·00	0·92	34·90
From 5–12 g. per day	5·93	5·38	48·38	4·66	11·48	10·58	11·96	0·81	35·79

[1] An approximation derived from the final weight of the excised primary growth and the period of time from the injection to the excision. The weight of tumour tissue in the bird in the form of metastatic deposits is not allowed for, although in some cases considerable.

From a general survey of the analyses as a whole, it must be acknowledged that there is more evidence to be derived from them in favour of the opinion that the higher proportion of hexone bases in malignant growths is dependent on the amount of nuclear substance present, than there is in support of the view, held by some, that this fact constitutes a specific property of the cancer cell.

Turning now to the individual amino-acids which have been estimated, it is felt that the accuracy of these estimations is not of a sufficiently high degree to warrant any definite deductions. On the other hand, if it is assumed that the errors of determination are of such a character that the results yielded by them may be used for a roughly comparative study, then one or two points of interest are noticeable. Firstly the values for lysine are higher in both the human and the chicken tumour proteins than they are in the normal tissue proteins.

Now it is quite true, as Kocher has remarked, that the recent work of Osborne and Mendel, and also of McCollum and Davis, has taught us the great importance of lysine for the growing animal, but it is hardly justifiable on these grounds alone to connect the high lysine content of tumours with their rapid growth.

On the one hand the increase of this particular diamino-acid in these cases —or indeed the increase of the diamino-acids generally—may merely represent the much greater activity of the cancer cell, expressed in its increased rate of cell-division and its consequent richness in nuclear substance; whereas on the other hand there is the view, apparently held by some, that one or more of these hexone bases acting as what Kocher calls a "growth-factor' may actually be the cause of cell-proliferation. The latter alternative seems altogether untenable, although at the same time it must be borne in mind that there seems every reason to believe that the growth of tumours is controlled by very much the same factors as influence normal growth.

These factors are many in number, for it is realised now that, in order to be adequate for growth, the diet of a young animal must be satisfactory from several standpoints. Thus, not only must the supply of protein in the diet be ample for the nutritive needs of the animal as regards its nitrogen intake, but it must also be satisfactory as regards its amino-acid make-up; while there is also the group of substances which are classed as "accessory growth hormones" which play such an important part in nutrition, but the nature of which we are at present quite unable to define.

All these factors, and no doubt many others, probably influence tumour growth to a greater or less extent, but it seems unwise to infer that any one of them, merely because it occurs in malignant growths to a greater extent than in normal tissues, is the underlying cause of an abnormal rate of cell-division.

Although the average results for all the tumours gives a higher figure for lysine than in the case of the normal tissues, this is not so when the analyses of the normal and pathological breast tissues are surveyed, and again no greatly increased amount of this amino-acid is present in the rapidly growing tumours as compared with the more slowly growing ones.

Of the other amino-acids, high figures for histidine will be observed in some cases, although it is realised that the doubtful accuracy of the arginine determinations has had the effect of yielding higher values for histidine than actually exist, in nearly every case.

Hopkins [1916] has recently demonstrated that this latter amino-acid, together with arginine, is to be regarded as of as much importance to the growing animal as is lysine. His studies have shown that either one of these two compounds can act as the precursor of the purines synthesised in the growing organism, so that they must both be of the greatest importance in the elaboration of nuclear substance.

We may on these grounds designate either histidine or arginine "growth-factors" with quite as much justice as Kocher has done in the case of lysine, although there is little evidence that they are markedly increased in cases of cancer.

The high figures for histidine, to which reference has been made, probably have their origin in at least two facts; firstly, there is a large amount of nucleo-protein in the product analysed, and secondly there is the presence of a certain amount of blood proteins in most of the tumours.

The Rous chicken sarcoma closely resembles the true rapidly growing sarcomata in being exceedingly vascular in character, so that all the growths examined contained a considerable amount of blood. Particularly in the case of the more rapidly growing examples was this so, for it was usual to find these tumours necrotic in the centre, and the cavity filled with extravasated blood and a thick mucoid fluid.

It is therefore conceivable that the high histidine figures may be partly due to the inclusion of a greater or less proportion of blood in the analysis.

Some evidence in favour of this explanation is given by the analysis of the spleen where a similarly high value for this particular determination is recorded.

Returning to a general survey of the results as a whole, it will at once be admitted that the conclusions put forward by Kocher have received no confirmation from this research. Seeking for an explanation of this, we find it in the reliance which this worker has placed on the analysis of normal tissues made by other workers.

Kocher's analyses of tumour proteins are in very good agreement with the analytical data recorded in this communication, as well as with those of Bergell [1907], so that it seems fair to assume that the analytical results for normal tissues which he quotes are inaccurate.

Below are tabulated as far as possible the available analytical figures given by various workers for some normal tissue proteins (Table X). It will be seen that the majority of the figures are in agreement in recording a higher percentage of diamino-acids in these tissues than is given by Wakeman.

In conclusion, it can be said that this research has yielded many results which support the view of Wells [1914], who holds that the cancer cell is very similar, if not identical, in chemical composition with the normal cell of the same type.

All the evidence points to the conclusion that the higher percentage of diamino-acids which is often present in the proteins of malignant growths is due to the greater proportion of nuclear material which they contain, for similarly high figures for this fraction of the protein molecule are found in the analysis of those normal tissues which possess cells rich in nuclein, as for example, normal human pancreas and spleen.

A similar explanation is applicable to the high diamino-acid content of spermatozoa, which is quoted by Kocher as an example of a germinating tissue rich in hexone bases.

TABLE X.

Analyses of Proteins of Normal Tissues. (Percentage of Total N.)

Tissue	Amide N	Humin N	Mon-amino N	Arginine N	Histi-dine N	Lysine N	Total di-amino N	Observer
Muscle, chicken ...	7·45	2·73	59·85	—	—	—	30·00	Osborne and Heyl [1908]
Muscle, ox	5·50	2·58	64·40	—	—	—	27·23	Osborne and Jones [1909]
Muscle, ox	—	—	—	7·50	1·80	7·60	16·90	Koelker and Slemons, quoted by Kocher [1915]
Muscle, chicken ...	6·63	1·76	61·55	11·10	8·45	9·81	30·27	Drummond
Muscle, human ...	6·30	2·30	62·25	11·20	4·26	12·65	29·90	,,
Liver, human ...	—	—	—	6·24	1·88	6·07	14·19	Wakeman [1908]
Liver, human ...	6·12	3·32	58·20	10·50	8·20	11·76	30·05	Drummond
Liver, chicken ...	8·18	5·53	59·47	9·30	7·30	9·30	27·60	,,
Liver, dog	—	—	—	8·32	2·31	4·81	15·44	Wakeman
Kidney, dog ...	—	—	—	4·19	2·51	6·05	12·75	,,
Kidney, human ...	5·05	3·24	64·40	11·95	5·89	7·00	25·85	Drummond
Haemoglobin ...	5·24	3·60	59·90	7·70	12·70	10·90	31·30	van Slyke [1911]
Serum protein, ox ...	7·05	1·60	58·80	11·05	4·40	13·55	31·75	Hartley [1914]
Serum protein, horse	7·25	1·60	61·95	9·50	5·60	12·80	30·00	,,

SUMMARY.

1. The distribution of nitrogen in a large number of the proteins of human and chicken tumours and normal tissues has been examined. A higher proportion of the diamino-acids generally exists in the case of the tumour proteins.

2. It has been shown that in all probability the diamino-acid content of a tissue varies directly with the amount of nuclear material present.

3. The more rapidly growing and cellular tumours therefore show a higher percentage of these substances than do the slowly growing ones.

4. There is no evidence from the analytical data recorded for the proteins of malignant tumours, which supports the view that the hexone bases are in any way responsible for an increased rate of cell-division.

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XXXIX. THE INFLUENCE OF FLESH-FEEDING ON URINARY CREATININE.

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Previous Work on the Conversion of Creatine to Creatinine.

In recent years a considerable amount of work has been done to ascertain whether either a conversion of exogenous creatine to creatinine or the converse takes place within the body.

It is generally agreed that creatinine given by the mouth or injected hypodermically is excreted as such to, at least, 80 %, and that there are no indications of its conversion to creatine [Myers and Fine, 1913, 1915; van Hoogenhuyze and Verploegh, 1905].

The question of the conversion of creatine to creatinine however, must still be considered unsettled. Czernecki [1905], Folin [1906], Klercker [cited by Folin, 1906], Lefmann [1908], and Folin and Denis [1914] found no evidence of a conversion. On the other hand, Pekelharing and van Hoogenhuyze [1910], Myers and Fine [1913, 1915], Towles and Voegtlin [1912], and van Hoogenhuyze and Verploegh [1905] found evidence of a conversion, which, however, is small in amount, being in most cases under 5 %, the rest of the creatine being either excreted unchanged or remaining unaccounted for.

Some workers (*e.g.* Levene and Kristeller [1909], McCrudden [1912] and Taylor [1915]) have published results of experiments in which importance is attached to variations in the excretion of creatinine while the subjects of the experiments were upon a flesh-containing diet.

These have either ignored the possible influence of meat-eating on the creatinine excretion, or have assumed, from the work of Folin [1905], that creatinine is of purely endogenous origin, and that its regular excretion is quite unaffected by the ingestion of creatine in any form.

Work done on rabbits is of doubtful value and need not be considered. The daily fluctuations of the excreted creatinine due to the impossibility of obtaining an exact twenty-four hour's secretion of urine, are too great in view of the small amount of conversion which is claimed to be obtained.

Only experiments, in which creatine was given to a subject on a creatine-free diet are worthy of attention.

The most extensive research on this subject is probably that of van Hoogenhuyze and Verploegh [1905], who, in a series of twelve experiments, took each 2 g. of creatine by the mouth, and concluded that creatinine appeared in the urine in small quantity.

An analysis of these results does not seem to bear out this conclusion. One of the six experiments on Verploegh is omitted as it is admittedly in error; and one on van Hoogenhuyze is omitted because a subsequent rise in the creatine excretion, not connected with the experiment, makes comparison impossible.

From 14th September to 3rd October the average creatinine excretion of Verploegh, excluding the days on which the creatine was taken, was 1·917 g. The average of the three creatine days and the following days was 1·905, a decrease of 0·012 g. For van Hoogenhuyze, the figures for the same period were—non-creatine days 1·886 g.; creatine and subsequent days 1·881 g., a decrease of 0·005 g. From the 25th July to 15th February Verploegh's average excretion, excluding creatine days, was 1·996 g. On the two creatine and following days the average was 1·988 g., a decrease of 0·008 g. From 25th January to 9th February van Hoogenhuyze's figures were—non-creatine days 1·886 g.; creatine and subsequent days 1·881 g., a decrease of 0·005 g.

From 25th January to 9th February van Hoogenhuyze's figures were —non-creatine days an average of 1·842 g.; creatine and following days 1·871 g., an increase of 0·029 g.

In view of the amount of creatine taken—2 g.—and of the daily fluctuation in the excretion, the results are not such as would lead to the conclusion that the conversion of creatine to creatinine occurs in the body.

These authors, and the others who conclude that creatinine is a derivative of creatine appear to have been unduly influenced by the work of Gottlieb and Stangassinger [1907, 1908], who believed that they had found in the liver, and to a lesser degree in other organs, an enzyme which converted creatine to creatinine. Valid objections to the acceptance of their results were raised by Mellanby [1908] and by Twort and Mellanby [1913], who showed that the chemical conditions and presence of bacteria were sufficient to account for the changes observed, and who, under strictly aseptic conditions, were unable to confirm their results. Dakin [1907] could find in the extract of liver, kidney, or duodenal mucous membrane no ferment acting upon creatine. It is interesting that Rothmann [1908] who repeated the work of Gottlieb and Stangassinger, found on one occasion almost twice as much creatinine as could be accounted for by the creatine added.

In any case, the utmost caution should be exercised in drawing far-reaching conclusions from such autolytic experiments, as we do not know that the chemical changes, which occur in an isolated organ in the process of dissolution, are strictly analogous to those that take place in the living body, where the chemical action may be influenced by the activities of other organs of the body.

The influence which an alleged creatinase exerts in converting creatine to creatinine within the body may be disregarded until more certain proofs of its existence are forthcoming.

Though the evidence that creatinine is derived from creatine is wanting, it appears likely that an intimate connection exists between these bodies. This view is indicated by their close resemblance in chemical composition; their mutual connection with the metabolism of muscle [Spriggs, 1907; Shaffer, 1908; Brown and Cathcart, 1909; Pekelharing, 1911]; and by the fact that in the urine of the bird creatine replaces the creatinine found in the urine of mammals [Paton, 1910, 1, 2; Paton and Mackie, 1912].

EXPERIMENTAL.

At the suggestion of Professor Noël Paton, this research was undertaken to ascertain to what extent, if any, the excretion of creatinine is affected by the eating of flesh, and to obtain further evidence of the conversion or non-conversion of ingested creatine to creatinine.

Some of the previous workers used in their experiments flesh, which contains from 3 to 4 % of creatine, while others used pure creatine. In the present series of experiments the influence of flesh and of creatine were investigated separately.

The subjects of the experiment, normal healthy males in the third or fourth decade of life, were put upon a creatine-free diet for several days. Then, on a given day, a weighed amount of flesh or of creatine was added to the diet, after which the creatine-free diet was continued.

Creatine and creatinine were estimated by Folin's colorimetric method, and the total nitrogen by Kjeldahl's method.

The results are recorded below:

A. *The influence of Flesh Feeding.*

TABLE I.

	Amount of urine cc.	Total nitrogen g.	Creatinine g.	Creatine g.	Remarks
Exp. I.	1840	13·22	1·41	—	Creatine-free diet
D. B.	1690	10·53	1·40	—	Creatine-free diet
	1897	15·13	1·50	0·06	227 g. uncooked meat in diet (*i.e.* 0·68 g. creatine)
	1300	11·88	1·43	0·01	Creatine-free diet
	1500	11·55	1·41	—	Creatine-free diet
Exp. II.	1510	11·74	1·63	—	
D.B.	1545	12·32	1·61	—	
	1150	13·17	1·65	0·1	114 g. uncooked meat in diet (0·34 g. creatine)
	1495	13·19	1·64	0·018	
	2290	11·91	1·61	0·02	
Exp. III	770	13·1	1·45	0·04	Creatine-free diet
M. C.	645	12·4	1·48	0·167	227 g. meat cooked
	850	12·2	1·40	0·200	Creatine-free diet
	700	11·5	1·35	—	Creatine-free diet
Exp. IV	1150	12·6	1·6	—	Creatine-free diet
N. M.	900	11·7	1·62	—	Creatine-free diet
	1100	12·9	1·86	0·001	227 g. meat (cooked)
	1100	12·9	1·64	0·002	Creatine-free diet
	900	11·7	1·46	—	Creatine-free diet

In the foregoing experiments, while the diet was creatine-free, except on the days when meat was given, it was only approximately fixed in amount and in composition.

In the two following experiments a fixed diet of known composition was maintained. In Exp. V, it consisted of oatmeal, pease-meal, white bread, dried skimmed milk, sugar, butter, and cocoa, and contained, approximately, protein 63 g., fat 80 g., and carbohydrate 320 g.

It was continued for six days. On the seventh day 125 g. of meat were added to the diet. An analysis of 125 g. of the same flesh was made by the method described by Brown and Cathcart [1909]. There was found 0·38 g. of creatine and no creatinine. The meat was eaten raw to avoid the possibility of a conversion to creatinine taking place in the cooking. Observations made by Noël Paton show this to occur to a small and varying extent[1]. In Exp. VI the pease-meal of the diet was replaced by bread. Table II gives results of the urinary analysis.

TABLE II.

	Amount of urine cc.	Total nitrogen g.	Creatinine g.	Creatine g.	Remarks
Exp. V	1017	10·35	1·46	—	Fixed creatine-free diet
J. B. O.	1360	10·46	1·52	—	Fixed creatine-free diet
	1400	10·08	1·44	—	Fixed creatine-free diet
	960	12·52	1·62	0·05	125 g. uncooked meat extra (0·38 g. creatine)
	1184	13·60	1·47	—	Fixed creatine-free diet
	757	10·96	1·32	—	Fixed creatine-free diet
Exp. VI	1000	8·90	1·70	—	Fixed creatine-free diet
J B. O.	710	9·72	1·77	—	Fixed creatine-free diet
	680	9·99	1·85	—	170 g. uncooked meat added to diet (0·5 g. creatine)
	685	9·61	1·60	—	Creatine-free diet

B. *The ingestion of creatine.*

One experiment on the ingestion of pure creatine was made.

The diet consisted of the same ingredients as in Exp. V, and contained approximately of protein 60 g., fat 79 g. and carbohydrate 315 g. It was continued for ten days. On the third, sixth and ninth days 0·5 g. of creatine was taken dissolved in water. The creatine used was a preparation of Merck's.

(It was analysed and found to contain a trace of creatinine.)

Table III gives the record of the urinary analysis.

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[1] We are indebted to Prof. Noël Paton for the following figures which indicate the varying extent to which cooking causes the conversion of creatine to creatinine in flesh.

Material	Total creatinine %	Creatinine %	Creatine %
Salt beef cooked	0·234	0·109	0·125
Horse-flesh cooked at knacker's	0·244	0·047	0·197
Mince cooked at home ...	0·260	0·096	0·164
Steak cooked in laboratory			
[Raw]	0·346	0·0194	0·3266
Grilled	—	0·0262	0·3198
Stewed	—	0·0210	0·3250
Boiled	—	0·0217	0·3243
Steak	0·315	0·0214	0·2936
Valentine's meat juice	0·696	0·338	0·358

TABLE III.

	Amount of urine cc.	Total nitrogen g.	Creatinine g.	Creatine g.	Remarks
Exp. VII	980	8·74	1·78	—	
J. B. O.	670	8·71	1·8	—	
	640	7·96	1·8	—	
	890	8·68	1·77	0·03	0·5 g. creatine
	760	8·71	1·8	—	
	730	8·71	1·808	—	0·5 g. creatine
	660	8·46	1·8	—	
	790	10·46	1·8	0·2	0·5 g. creatine

DISCUSSION OF RESULTS.

A. *Flesh.* In every instance, there follows on the ingestion of the meat an increased excretion of creatinine, which is confined chiefly to the day on which the meat is eaten. The following table summarises the series, showing the average in the preliminary days of creatine-free diet and the amount excreted on the day on which the meat was taken.

TABLE IV.

Creatinine in grams.

Exp.	Subject	Preliminary period average	Day of meat ingestion	Increase	Amount of meat ingested
I	D. B.	1·405	1·5	0·095	227 g. raw
II	D. B.	1·62	1·65	0·03	114 g. raw
III	M. C.	1·43	1·48	0·05	227 g. cooked
IV	N. M.	1·61	1·86	0·25	227 g. cooked
V	J. B. O.	1·47	1·62	0·15	125 g. raw
VI	J. B. O.	1·735	1·85	0·115	170 g. raw

It is seen that there is no parallelism between the amount of flesh eaten and the increase in the excretion of creatinine. The results show that estimations of the excretion of creatinine, while a subject is upon a flesh-containing diet, are quite devoid of value, even though the amount of flesh in the diet be kept constant.

B. *Creatine.* Of the occasions on which pure creatine was given, the first shows a decrease in the output of creatinine of 0·03 g.; the second a slight increase of 0·008 g., and the third, no change. These small variations are within the limits of the normal fluctuation. This experiment appears to afford no evidence that ingested creatine is converted within the body to creatinine.

Why, then, is there an increased creatinine output after a flesh diet, and why does not a similar increase take place after the ingestion of pure creatine? There are at least, three possible explanations:

(*a*) Creatine (or creatinine) may be "protected" when administered in flesh from destruction in the alimentary canal, and so a greater absorption of these bases may take place. Twort and Mellanby [1913, p. 45] have shown that flesh may "protect" added creatine from destruction (*in vitro*) by certain creatine-destroying bacteria of the intestinal canal. They suggest that this bacterial action is the cause of the loss of creatine and the retention of creatine nitrogen when pure creatine is given by the mouth to a subject on a creatine-creatinine-free diet with a moderate or low nitrogen intake. Against this must be put the finding of MacNeal and Chace [1913], viz. that, under normal conditions, the upper portion of the gut is comparatively sterile. Myers and Fine [1915] urge that, in any case, the loss of creatine by bacterial action, would be inappreciable, because of the rapid rate of absorption.

(*b*) In addition to the preformed extractable creatine, another precursor of creatinine may exist in flesh which after absorption, at a later stage of metabolism, may yield creatinine. One of us [Burns, 1916, 1] has shown that in the developing chick guanidine is formed in muscle, in point of time, before creatine and reaches its highest concentration at the point when creatine appears in detectable amounts. Further, the administration of the salts of guanidine [Burns, 1916, 2] may produce a small increased daily output of creatinine per kilo. body weight. Flesh contains not only creatine (or some bodies which post-mortem give rise immediately to creatine and more slowly to creatinine) but free guanidine, methyl-guanidines and complexes which are readily oxidised to guanidine. It might well be that under different conditions of oxidation, *e.g.* at different stages of metabolism these various catabolites might be produced [Ditman and Welker, 1909].

(*c*) The increased creatinine excretion after the ingestion of flesh may be due to the specific dynamic action of the various products of flesh catabolism. It is well known that any factor which increases the heat production of an animal increases the creatinine output, *e.g.* fevers [Leathes, 1907] and artificial hyperthermia [Myers and Volovic, 1913].

The increased creatinine output got by Myers and Fine [1915] after the ingestion of pure creatine may possibly be explained as the result of the diuresis. Burns and Orr [1914] pointed out that the production of a diuresis by the ingestion of water markedly and immediately raised the creatinine coefficient. The administration of 1 g. of creatine (in 250 cc. of warm water) to Myers increased the volume of urine from 570 to 910 cc. and produced no increase in the creatinine or creatine of the urine. The addition of 2 g. of creatine raised the urine volume to 1330 cc. and its creatinine content by 0·03 g. The same amounts of creatine taken by Fine resulted in urinary alterations of water 645 cc. to 1100 and 1205 cc.; of creatinine 1·29 to 1·26 and 1·32 g. (20 and 29 % of the ingested creatine was eliminated as such in this case.)

Burns and Orr [1914] obtained similar increases in the creatinine output on the ingestion of water.

		Creatinine g.		Creatine g.	
Exp.	Amount of water	Before	After	Before	After
III.	3 litres	1·31	1·33	—	—
VII.	,,	1·33	1·36	0·01	0·01

SUMMARY.

1. The ingestion of flesh causes an increased excretion of creatinine and generally the appearance of creatine in the urine.

2. The amount of the increase is not proportional to the amount of flesh taken.

3. Although cooking increases, to a variable extent, the amount of creatinine present free in flesh, cooked meat does not necessarily cause a greater increase in urinary creatinine than uncooked flesh.

4. Pure creatine administered per os is only to a slight extent eliminated as such and does not markedly alter the creatinine excretion.

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XL. METHODS FOR THE ESTIMATION OF MIXTURES OF FOUR OR MORE CARBOHYDRATES, INVOLVING OXIDATION WITH BROMINE.

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In the course of investigations upon the diurnal changes in the carbohydrate content of green plant tissues, many analytical difficulties were encountered, and it was therefore decided critically to examine existing methods and to devise others if possible. The work was unavoidably delayed and since there is but little prospect of bringing it to completion in the near future, we have decided to publish what has been outlined up to the present. While carrying out this work a series of papers has appeared by Messrs Davis and Daish [1913] and their collaborators, dealing with the same subject and we have been much indebted to them.

THE CARBOHYDRATES OF THE GREEN LEAF.

The five carbohydrates usually found in foliage leaves are starch, sucrose, *d*-glucose, *d*-fructose and maltose, and in addition to these sometimes small quantities of pentoses are known to be present. The estimation of starch has been fully discussed by Davis and Daish [1914] and that of the pentoses by Davis and Sawyer [1914] and by Daish [1914]. In the present work, therefore, only the remaining four sugars will be considered.

REDUCTION METHOD.

Before beginning this work, it was necessary to find a suitable method of quantitatively estimating reducing sugars gravimetrically. As all methods based on the use of Fehling's solution are liable to error, owing to the solvent action of the hot sodium hydroxide on cuprous oxide, it was decided after careful tests to use Kendall's method [1912], weighing the copper as cupric oxide as advocated by Davis and Daish [1913] and by Zerban and Naquin [1908], the action of reducing gases being avoided by heating the Gooch crucible containing the precipitate inside a larger porcelain or nickel crucible.

It has been stated by some that cupric oxide is hygroscopic and that this constitutes an objection to the method. However we have found no trace of this hygroscopic property throughout the numerous experiments carried out during this research, provided the carbonate used was pure. We have noticed, however, that if there is any iron present as an impurity in the carbonate used for reductions, then hygroscopic properties are observed, apparently in the cupric oxide, in reality due to the presence of red oxide of iron (Fe_2O_3), and moreover in such cases, we observed that the cuprous oxide was precipitated yellow whereas in all other cases it came down red. The blue amphibole asbestos for use in the Gooch crucibles must also be treated as insisted on by Davis and Daish, in order to ensure its constancy in weight when exposed to hot alkaline solutions.

Kendall's procedure, very briefly described, consists in adding to the sugar solution which has been made up to 100 cc. with distilled water, solid salicylic acid, a solution of copper sulphate and a strong solution of potassium carbonate in the order named[1]; the mixture having been gently shaken is heated in a rapidly boiling water-bath for 20 minutes. Boiling should begin again within from one to two minutes of the introduction into the bath of the cold solution. Kendall has prepared tables for the reducing powers of glucose, invert sugar, maltose and lactose, but since a table for fructose was not included, one has been prepared by us and used for our analyses [1916].

ANALYSIS OF SUCROSE, MALTOSE, *D*-GLUCOSE AND *D*-FRUCTOSE.

(1) *Sucrose.*

The estimation of sucrose is usually carried out by inversion under Clerget conditions or by some appropriate modification of the method. When, however, maltose is present treatment with a mineral acid results in hydrolysis of a portion of this sugar. To avoid this citric acid in from 2 % to 10 % solution has been employed. Davis and Daish [1913] have studied this point carefully and recommend the use of invertase in preference, or at least as a check upon, hydrolysis with citric acid. The invertase may most conveniently be prepared and preserved according to Hudson's [1914] directions.

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[1] The amounts employed are: salicylic acid 5 g., copper sulphate 15 cc. of a solution containing 133·33 g. of pure crystals per litre, and potassium carbonate 25 cc. of a solution containing 600 g. of anhydrous salt per litre.

The calculation of the amount of sucrose is based upon the readings of the polarimeter before and after inversion or upon the copper reducing power of the solution. The changes in these two values should of course give concordant results; but sucrose itself has a slight reducing power when mixed with reducing sugars, so that a slight discrepancy will usually be observed.

When the amount of sucrose has been determined the rotation due to it may be calculated and hence the rotation of the remaining three reducing sugars in the mixture. One other sugar has now to be determined directly (as a solution of equations containing three unknowns is not satisfactory). The most usual sugar to determine now is maltose, and then by subtracting the rotation due to sucrose and maltose from the initial observed rotation and deducting the amount of copper due to maltose from the observed copper reducing power of the original solution, there remain two equations for the two unknowns, glucose and fructose, which may easily be found.

Now maltose may be estimated either by Brown and Morris's method [1893], of hydrolysis with hydrochloric acid under standard conditions, or by Davis and Daish's method [1913] of fermentation with pure cultures of maltase-free yeasts, which seems to have given very accurate results in the hands of its originators. But as each estimation requires at least twenty-one days and involves the use of a pure culture of a particular yeast it must be conceded that it is cumbersome. Accordingly a method is here suggested whereby fructose and not maltose is determined directly in the remaining mixture of three sugars. This method is outlined in the following pages.

(2) *Oxidation methods in acid or neutral solution.*

In all cases in which it is desired to estimate the separate sugars in a mixture of glucose, fructose and maltose, it is necessary as previously pointed out either to have three equations or to have a means of estimating one sugar by some special method.

At the outset it seemed that use might be made of the differential action of bromine upon the ketonic and aldehydic sugars. The former are scarcely oxidised at all, whereas the latter readily yield aldonic acids.

Bunzel [1909] has carefully studied the mechanism of the oxidation of glucose by bromine. He found that the amounts of acid and of bromide produced, as well as the amounts of glucose and of bromine disappearing, are equal to those calculated on the hypothesis that one molecule of bromine oxidises one positive glucose ion to gluconic acid. Thus when acidified with hydrochloric acid, 0·1 normal, the equation is as follows:

$$C_6H_{13}O_6^+ + Cl^- + Br_2 + HOH = C_6H_{12}O_7 + HCl + 2HBr.$$

The correctness of this view is supported by the constancy of the velocity constant, K, in the following equation, even under widely varying conditions of acidity and with different concentrations of bromine and glucose·

$$\frac{dx}{dt} = K . C_{C_6H_{13}O_6^+} \times C_{OH^-} \times C_{Br_2\,(free)}.$$

From this it is seen that the rate depends upon three factors: (*a*) the concentration of the positive glucose ion $C_6H_{13}O_6^+$; (*b*) the concentration of the hydroxyl ions; (*c*) the concentration of the free bromine in solution.

Bunzel has shown that provided the concentration of the hydrogen ions is over 0·1 normal the hydroxyl ion factor may be entirely neglected. The velocity under these conditions is proportional to two variables, the concentration of the glucose and of the free bromine. Bunzel has also proved that in an acid medium the positive glucose ion goes quantitatively into gluconic acid. This may be expected to give rise to its lactone which is further oxidised by bromine very slowly.

The oxidation of the negative glucose ion $C_6H_{11}O_6^-$ probably yields a large number of acids. These have been studied by Nef [1907, 1910, 1914]. Since glucose is a very weak acid its dissociation in this manner is almost completely prevented by strong acids, with which it forms oxonium salts and ionises to $C_6H_{13}O_6^+$ as previously mentioned.

From these considerations it is evident that in 0·1 normal acid saturated with bromine and maintained in this condition, the rate of oxidation of glucose will be that of a unimolecular reaction. For the other variable, the concentration of bromine has now been reduced to a constant. This being so the same fraction of the glucose present will be oxidised to gluconic acid in a given time, irrespective of the initial concentration, provided the temperature is maintained constant.

The reaction under these conditions is a perfectly definite one and should be available for the estimation of glucose. The standard conditions will be discussed again in relation to the actual procedure adopted.

The action of bromine upon maltose in the cold is quite similar to that upon glucose, maltobionic acid being the product. Fructose is, however, usually considered to be almost unaffected by this halogen. At the outset it appeared possible to remove the glucose and maltose quantitatively by oxidation with bromine. The reducing power of the solution would then have been due to fructose alone. Direct experiment, however, has shown that this is not rigorously exact, for when sufficient time has elapsed for the oxidation of all the maltose a small proportion of the fructose has in some way been destroyed. Moreover the oxidation of the glucose is never complete, or to be more accurate, prolonged treatment with bromine always leaves a solution which has still some reducing action upon Kendall's solution, corresponding to about 2 % of the glucose originally present. With maltose too the residual reducing power after treatment with bromine, though negligible, is quite perceptible.

This residual reducing power of glucose solutions most probably has its origin in the formation of gluconic lactone from the first formed gluconic acid as its concentration increases. That this is the case is borne out by the fact that all our attempts to isolate the phenylosazone of glucose from such solutions were unsuccessful, giving rise only to the production of gluconic phenylhydrazide. Moreover two experiments performed under the standard conditions and at average temperatures differing by about two degrees showed that oxidation by bromine for 66 hours afforded a solution with a larger residual reducing power than a similarly conducted oxidation for 42 hours. This apparently indicates the formation of the lactone on prolonged oxidation. A similar explanation is most likely applicable to the very slight residual reducing power observed also in maltose solutions.

Details of these analyses are given in the experimental portion of the paper.

The action of bromine upon these three sugars was studied at temperatures between 0° and 35° before all hope of a very exact separation was abandoned.

Since, however, the action may be reduced to the monomolecular type by maintaining the bromine in saturated solution, and by having a suitable excess of mineral acid, it is obvious that constants can be determined for each sugar, representing the fraction of its initial reducing power remaining after treatment with bromine under standard conditions. Accordingly such constants were found for experiments carried out at room temperature, after an attempt to work at 0°; the latter steady temperature was not suitable since the solution became of indefinite composition owing to the separation of very large amounts of bromine hydrate. The solutions were shaken continuously to ensure complete saturation with bromine throughout the period of oxidation. Shaking was effected by rotating the bottles, which were partly filled with glass beads, about a horizontal axis. To ensure a more uniform temperature they were placed in a tank filled with water, and covered to exclude the light. A small electric motor running at about three thousand revolutions a minute was geared down suitably to rotate the solutions.

The equations available for determining the composition of a mixture of three sugars are then those usually employed for polarisation and reduction, with a third similar to the reduction equation but introducing a constant for residual sugar after halogen oxidation, thus:

$$(1) \quad 100\,[\alpha]_D^{20} = x\,[\alpha]_{D_g}^{20} + y\,[\alpha]_{D_f}^{20} + z\,[\alpha]_{D_m}^{20},$$

where x, y and z represent the amounts of glucose, fructose and maltose respectively.

$$(2) \quad w_1 = k_g x + k_f y + k_m z,$$

where k_g represents the weight of cuprous oxide obtainable from Kendall's solution by 100 mg. or other suitable quantity of glucose; k_f and k_m have similar meanings.

$$(3) \quad w_2 = k_g . a . x + k_f . b . y + k_m . c . z,$$

where a, b and c are the percentages of residual sugar after treatment with bromine under the standard conditions.

On substituting the experimental data in the above equations an attempt was made to solve for the three unknowns; but in such methods of solution experimental errors are often largely multiplied and the magnitude of the errors so introduced in the mathematical operations was found in this case to be so great as to render the method useless.

Now, when the time allowed to elapse was 66 hours at room temperature (17°–18°) the values for the residual sugar constants were $a = 0{\cdot}033$; $b = 0{\cdot}970$; $c = 0{\cdot}006$. It will be noticed that the sum of these constants is 1·009, from which it follows that it is very nearly correct to assume that no fructose has disappeared, but that the other two sugars have been completely oxidised away.

It has been found that part at least of the small loss of fructose is due not to its oxidation by bromine but to its decomposition by the decinormal acid added to the solution, and further reinforced in the case of mixtures of the three sugars by the hydrobromic acid generated in the course of the reaction. Experiments proving this are shown in the experimental portion of this paper.

In order to test by direct experiment the supposition that under certain standard conditions it is legitimate to assume that all the residual reducing power of the solution is due to fructose when glucose and maltose have also been present at the start, solutions containing these three reducing sugars were made up. They were then examined by the polarimeter, and the reducing power both before and after oxidation with bromine was determined for suitably diluted aliquot portions of the original solutions by Kendall's method[1]. The results are shown in Table I. It must be remembered that, of the three, fructose is the only sugar which has been directly determined. The quantities of the other two had to be found by solving the simultaneous equations (1) and (2) for two unknowns. The multiplication of errors is not nearly so serious as with three unknown quantities, yet the accuracy to be expected is not of the same degree as in direct determinations.

TABLE I.

	I		II		III	
Temperature	13°–16°		17°–18°		10°–11°	
Time of oxidation	42 hours		66 hours		66 hours	
	Taken g.	**Found g.**	**Taken g.**	**Found g.**	**Taken g.**	**Found g.**
Fructose	0·7686	0·7650	0·4166	0·4150	0·4975	0·4615
Glucose	1·0260	0·9904	0·6955	0·5886	0·4910	0·4556
Maltose	0·8950	0·9863	0·6615	0·6953	0·4888	0·4778
Total	2·6896	2·7417	1·7736	1·6989	1·4773	1·3949

These results indicate that the method is capable of giving fairly accurate values for fructose in the presence of either or both of the other reducing sugars. The details of the estimation may possibly be improved so as to render the analysis of greater accuracy. In the latter quality this method is not so good as that of Davis and Daish but inasmuch as it can be carried out in three days instead of in three weeks, it may be found to commend itself for certain purposes.

(3) *Oxidation methods in alkaline solution*[2].

In alkaline solution the sugars are rapidly oxidised by atmospheric oxygen with the production of a brown colour. The relative rates of oxidation under these conditions are the same as in acetic acid, fructose being the most readily attacked. This difference between the reactions in acid and alkaline solution is most simply accounted for by the hypothesis that the sugars are weak acids, the ionisation of which is hindered by other acids whereas the sodium salts formed in alkaline solution are readily ionised. This results in the liberation of very unstable and easily oxidisable negatively charged sugar ions. Mathews [1909] noticed that the rate of oxidation of glucose and other sugars in alkaline solution increased as the reaction proceeded. He adduced reasons for the belief that the glucose molecule is broken up into a number of active reducing molecules under these conditions. This process takes place even in the absence of atmospheric oxygen, and so has no connection with any oxidation processes in which gaseous oxygen is concerned, though intra-molecular oxidations are not excluded.

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[1] The solutions for standard oxidation consisted of 50 cc. sugar solution diluted to 70 cc. and then 10 cc. normal sulphuric acid added and finally bromine 5 cc.

[2] This problem was forced upon our notice through the accidental addition of a slight excess of alkali in neutralising, as explained in the "Experimental" portion of this paper.

When alkaline solutions of the three reducing sugars glucose, fructose and maltose are exposed to gaseous oxygen they are readily oxidised as has already been mentioned. In consequence one might expect a decrease in their reducing power towards copper solutions. On testing this point directly it was found that the change in reducing power was an increase, followed by a decrease. In one case for example the procedure was as follows: a 2 % solution of each of the three sugars was mixed with an equal volume of normal sodium hydroxide. Before mixing, immediately after mixing and at various intervals of time the reducing powers of the solutions were determined by means of Fehling's solution. In the first case an equal volume of water was added to the sugar solution, in order that the analyses might be exactly comparable with the others of the series. The flasks were allowed to stand at room temperature, about 15°, and were not shaken up with air. At the same time in these preliminary experiments no precautions were taken to avoid oxidation. The results are shown in Table II.

Since there is a definite change in the reducing powers of the sugars when allowed to stand in half-normal sodium hydroxide it is evident that a set of constants can be obtained for the different sugars showing what alteration takes place in each under standard conditions. In obtaining these due regard must be paid to the most suitable times, temperatures and concentrations. Means must also be taken to prevent any appreciable amount of oxidation, for example by filling the flasks with hydrogen. If it so happened that all the reducing sugars were altered in the same proportions the effect would only be to alter the magnitudes of the usual constants for the reducing powers of these substances towards the various alkaline copper solutions in use. But experiment has shown that the change in the reducing powers of the various sugars takes place to very unequal extents, as after an initial increase, the rate of decrease is most rapid for fructose.

The results of these experiments are tabulated below; the concentrations recorded refer in each case to the mixture not to the original strengths of the separate solutions.

TABLE II.

Action of N/2 sodium hydroxide on sugars at room temperature.

Glucose 1 %			Glucose 3 %			Fructose 1 %			Maltose 1 %		
Time hrs.	mins.	Reducing power	Time hrs.	mins.	Reducing power	Time hrs.	mins.	Reducing power	Time hrs.	mins.	Reducing power
0	0	100·0	0	0	100·0	0	0	100·0	0	0	100·0
0	15	132·0	0	7	103·5	0	8	132·3	0	7	137·4
0	25	131·5	0	15	104·7	3	37	132·3	3	35	128·8
0	44	131·5	0	28	104·7	23	45	83·3	23	0	115·3
21	55	95·7	4	25	102·3	47	0	66·4	46	45	108·9
22	8	94·6	24	20	94·6	—		—	—		—
43	40	88·0	—		—	—		—	—		—

TABLE III.

Action of 2N sodium hydroxide on sugars at room temperature.

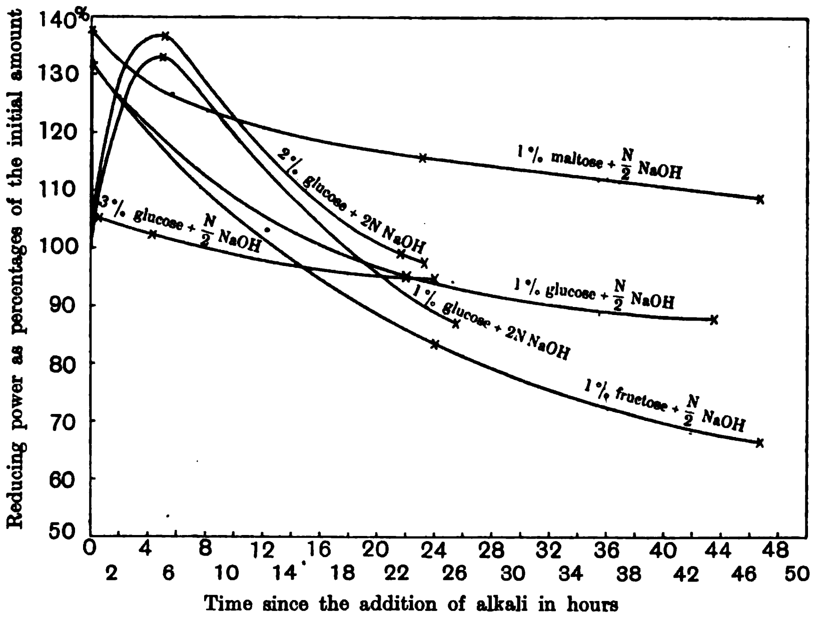
Glucose 1 %			Glucose 2 %		
Time hrs.	mins.	Reducing power	Time hrs.	mins.	Reducing power
0	0	100	0	0	100
0	5	113·2	0	7	104·4
4	50	117·0	5	47	151·1
24	20	88·5	23	50	95·3
126	30	34·0	—		—

The results are graphically shown in the figure.

Considering the N/2 alkali, with the 1 % solutions there is a very rapid rise in reducing power consequent upon the addition of alkali. This rise is identical in magnitude for glucose and for fructose within the limits of accuracy of the method. With maltose a somewhat greater rise is found. It must however be pointed out that the hexoses are present in equimolecular proportions, viz. 10/180 normal (= 0·055 N), whereas the molecular concentration of the maltose is far smaller, 10/342 normal (= 0·029 N).

With 3 % glucose the increase in reducing power is far smaller, and attains its maximum value more slowly.

Mathews [1909] clearly showed that there was an increase in the number of active reducing molecules consequent upon the addition of alkali. This he ascertained by measuring the rate of absorption of oxygen by a glucose solution which was kept over mercury after being made alkaline. From the preceding tables it is evident that the increase is very considerable and a maximum is quickly reached. However the relationship between the quantity of alkali added and the percentage of sugar undergoing change requires further investigation. It has recently been found by Powell [1915] that sodium hydroxide reacts with glucose and fructose with the formation of sodium salts, each molecule of sugar taking up one sodium ion. This conclusion was deduced from the study of the retardation in the hydrolysis of ethyl acetate by the hydroxide, which follows the addition of a sugar.



Before becoming acquainted with Powell's results an attempt was made by us to obtain evidence concerning the union of sugar and alkali by means of conductivity measurements. The conductivity of 1 % glucose solution was measured—it was found to be about half that of the Dublin water supply—and also the conductivity of seminormal sodium hydroxide. These quantities were chosen as being those employed in obtaining the results shown in Table II, though as the sugar solution is only 0·055 normal it would perhaps have been advisable to use more dilute alkali. Then however a compound, if formed, would be almost completely ionised, whereas with one component in large excess such ionisation would be diminished. The objection to using a strong sugar solution is that the viscosity changes would then begin to have a serious effect on the conductivities.

The conductivity of a solution composed of alkali and glucose in the same final concentrations was then measured at various intervals in a closed vessel.

TABLE IV.

Solution		Conductivity in mhos at 0°, × 10^5
Glucose 1 % (0·055 N) ...		2·181
Sodium hydroxide 2 % (0·5 N)		5661·0
Glucose 1 % ... ... Sodium hydroxide 2 %	15 minutes after mixing	5272·0
„ „	55 minutes „	5124·0
„ „	105 minutes „	5121·0
„ „	22·5 hours „	5170·0
Sodium hydroxide 2 % taken fresh from the corked flask, after standing in it for 23 hrs.		5214·0

Now neglecting the slight effect that the alteration in viscosity due to the glucose will have upon the conductivity, and assuming that the glucose combines in unimolecular proportions with the hydroxide, a conductivity of $C \times 10^5 = 5095$ should result if the conductivity varied directly as the concentration. This value is in reality slightly too low, for the more dilute solutions have higher molecular conductivities, 0·45 N hydroxide conducting better relatively than does 0·50 N. However the increase in viscosity acts in the opposite direction. Thus a comparison of the observed conductivity after 105 minutes, $C \times 10^5 = 5121$, with the calculated conductivity, $C \times 10^5 = 5095$, points to the formation of a compound between the sugar and alkali. This takes place to the extent of 95 % of the theoretical amount, the compound being dissociated to the extent of 5 %, or taking the final conductivity as $C \times 10^5 = 5214$, the dissociation amounts to 21 %. On the whole however it would be very unwise to conclude that the changes in such an unstable reaction-mixture as an alkaline sugar solution could be accurately represented by any one reaction.

In concluding this section it may be remarked that though the study of the oxidation of the sugars in alkaline solution has not lent itself to any quantitative method for their estimation when two or more are present together, yet it has shown what grave errors may result if sugar solutions are allowed to become alkaline in the course of analyses. Such solutions should always be neutral or faintly acid.

(6) *Oxidation by cupric acetate in presence of acetic acid.*

The use of Barfoed's solution is well known as a qualitative test to distinguish glucose and fructose, which reduce this reagent, from maltose and lactose, which under the specified conditions are without this action. All attempts to use the method for quantitative work have failed, since the disaccharides effect some reduction when the boiling is continued sufficiently long to oxidise all the hexoses. The amount of acetic acid present is also an important factor, and during the heating some of it is driven off.

This reaction has been very carefully studied by McGuigan [1907], Mathews and McGuigan [1907] and by Bunzel [1908]. It was found by Bunzel that the reaction is of the fifth order. However it seemed possible that it might be reduced to the first order, by adopting certain standard conditions, and be then available for the analysis of mixtures by differential oxidation of the sugars. With this object sodium acetate, 10 g., was added to 100 cc. of Barfoed's solution[1] to ensure a constant acidity of small magnitude. A large excess of the reagent was also taken, 100 cc. for 25 cc. of a 2 % solution of glucose, so that the change in the concentration of the copper should not be of too considerable magnitude. Furthermore the boiling of the mixture was effected under a reflux condenser. The cuprous oxide was collected in a Gooch crucible and weighed as cupric oxide. The following table shows that the solvent action of the acetic acid upon the oxide is too great to allow of accurate quantitative estimations by the method. Even the addition of the sodium acetate did not suffice to reduce this to negligible proportions.

TABLE V.

Cupric oxide from 100 *cc. Barfoed's solution,* 10 *g. sodium acetate and* 25 *cc. of* 2 % *glucose, the latter being added cold to the former already heated to* 90° *in a water-bath. Solution cooled under water-tap at end of heating.*

Time of heating	Cupric oxide
10 min.	1·1296 g.
15	1·2773
20	1·2257

Furthermore it was found that when 1 % glucose was used, and the heating was continued for fifteen minutes, the yield of cupric oxide was 0·8234 g.; indicating a reducing power nearly half as great again as for a 2 % solution.

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[1] Copper acetate crystals 66 g. per litre, glacial acetic acid 10 cc. per litre.

A similar attempt using the mono- and di-hydrogen sodium phosphates instead of sodium acetate was also found to be unsatisfactory. However though this attempt at reaching suitable conditions is a failure it is perhaps worth recording to save others from similar waste of time.

EXPERIMENTAL.

The sugars used in the course of this investigation were the following: (*a*) for preliminary work, the pure anhydrous glucose, fructose (laevulose) and maltose of commerce; (*b*) for accurate work, determinations of constants, etc., Kahlbaum's sugars were used as a rule, the fructose being the "laevulose from inulin I." In some experiments pure commercial glucose and maltose were employed, after recrystallisation from alcohol three times.

The powdered sugars were kept in desiccators over sulphuric acid for long periods. The residual moisture was determined by heating a small portion, about 2 g., in an exhausted flask connected with drying tubes of phosphoric oxide at air temperature which led to a Geryk pump. The temperature at which the sugars were heated was that of the water-oven. When drying fructose it is advisable to use a lower temperature, about 50°, at first if the sample contains any appreciable amount of moisture. The "laevulose from inulin I" however did not melt and remained perfectly white when heated to 98° at atmospheric pressure in a vessel containing phosphoric oxide. Its purity was further tested by determining its specific rotation, in 5 % solution at 17·4° in a 20 cm. tube, using a Schmidt and Haensch polarimeter. When corrected for temperature it gave $[\alpha]_{20}^{D} = -92{\cdot}3°$, which agrees closely with the value chosen for use throughout this work, viz. $-92{\cdot}5°$. Later on a polarimeter by Adam Hilger was employed.

Anhydrous maltose was prepared by distillation with alcohol and benzene as recently described by us [Atkins and Wilson, 1915], but owing to its extremely hygroscopic nature it was deemed advisable to employ the hydrate for quantitative work, and to correct for the small excess of moisture beyond the theoretical amount of water of crystallisation.

Action of Bromine on Glucose, Fructose and Maltose.

To study this action solutions of the sugars were saturated with bromine, and allowed to stand in the dark over excess of the liquid halogen. In some experiments the bottles were shaken from time to time to maintain saturation. In determining constants the shaking was continuous.

At the end of the period chosen the liquid bromine was removed by means of a separating funnel and what remained over was converted into hydrobromic acid by passing in sulphur dioxide till the solution was nearly colourless. The last traces were destroyed by the careful addition of a solution of sulphurous acid from a burette. The mixture was then almost neutralised with solid potassium carbonate, taking care to leave the solution faintly acid at the end. A blank experiment showed that this procedure afforded a solution which had no reducing action on alkaline copper solutions.

The reducing power of the sugar solutions after treatment with bromine was determined by Kendall's method, but the cuprous oxide was oxidised by heating in air, and weighed as cupric oxide. The amounts of fructose were found from a table prepared by the authors in exact accordance with Kendall's method of drawing up tables for the other sugars [1916].

The following tables show the results of the preliminary experiments.

TABLE VI.

Oxidation of glucose by bromine in approximately saturated solution.

Temperature	Approximate percentage strength of sugar solution	Time	Percentage of glucose remaining. Initial concentration = 100 %	
Room 13°–16°	10	16 days	2·5	
,,	10	12 ,,	3·0	
33°	2	4 ,,	8·9	
0°	1½	90½ hours	50	
0°	1½	91¾ ,,	69·13	
0°	1½	90 ,,	34·77 35·76* 53·03†	solution shaken
	Same experiment			

TABLE VII.

Oxidation of fructose by bromine in approximately saturated solution.

Temperature	Approximate percentage strength of sugar solution	Time	Percentage of fructose remaining. Initial concentration = 100 %	
Room	12½	16 days	97	
,,	11½	12 ,,	98	
40°–45°	11	4 ,,	92	
22°	5	4 ,,	95	
0°	1	90 hours	100·0* 101·6†	solution shaken
	Same experiment			

TABLE VIII.

Oxidation of solution of maltose by bromine in approximately saturated solution.

Temperature	Approximate percentage strength of sugar solution	Time	Percentage of glucose remaining. Initial concentration = 100 %
Room	11	16 days	0·0
,,	12	12 ,,	0·0
33°	1½	4 ,,	2·3
0°	2	90¼ hours	37·4
0°	1	92 ,,	50·8
0°	1	90 ,,	19·50* 30·75† solution shaken
	Same experiment		

The figures marked with an * resulted from experiments where the solutions had been allowed to stand for four hours with a slight excess of potassium carbonate. Those marked with a † were given by the same solution after standing for two days. The solid carbonate was added to neutralise, and the slight excess varied in each case. The delay was accidental, only the glucose being estimated immediately after neutralisation in the case of these experiments carried out at 0° during 90 hours.

The action of alkali in bringing about an increase in the reducing power of the sugar solutions was here forced upon our notice for the first time. The phenomenon was studied at some length later on, and has been already mentioned in the earlier portion of this paper.

Inspection of the foregoing results shows that fructose is not appreciably oxidised at 0°, when allowed to stand for nearly four days. Prolonged action at higher temperatures appears to destroy some of this sugar.

It must however be noted that the experiments at 0° were carried out with "laevulose from inulin I," which is quite free from glucose. The others were performed with crystallised laevulose, Kahlbaum's also, and any small quantity of glucose it may have contained would have been oxidised and so cause the loss of fructose to appear too great. We suggest that the purity of samples of laevulose might be tested by allowing their solutions to stand with bromine at air temperature for one day or at 0° for four days. The loss of reducing sugar will then be due almost entirely to the destruction of glucose, maltose or other aldehydic sugar. Our experiments on fructose are in agreement with those of Votoček and Nemecek [1910]. They found that whereas, when exposed to the action of bromine for 24 hours at 21° only 5·10 % of *d*-galactose remained and 22·20 % of *d*-glucose, yet there was 100·00 % of *d*-fructose.

Action of dilute acid (N/8) on fructose.

On considering the results shown in Table I, it was thought that it might be of interest to determine what percentage of fructose apparently lost by oxidation in these experiments might have been really destroyed by the action of dilute acid on this sugar.

Accordingly to test this the following experiments were carried out, the procedure being exactly the same as in the standard experiments (Table I) except that no bromine was added. A certain portion of the sugar solution made by dissolving a weighed quantity of fructose in water was taken and the amount of sugar present determined, in one case by reduction by Kendall's method and in the other by polarimetric observations. Then 50 cc. of the original solution were diluted to 70 cc. and 10 cc. normal sulphuric acid added giving a solution of $\frac{1}{8}$ normal acidity which is the original strength of the acid in the oxidation experiments. This solution having been shaken to mix was allowed to stand for 66 hours after which period it was made up to a known volume, then an aliquot portion removed and the remaining fructose estimated by reduction. The percentage of fructose destroyed by N/8 acid under the above conditions was thus determined. It must be observed however that in experiments where a mixture is being quantitatively oxidised the acidity is greater than $\frac{1}{8}$ normal owing to the production of acid in the usual way when halogen oxidation occurs and moreover owing to aldonic acids produced from the sugars.

The results of these experiments are shown in Table IX where the figures represent the weight of fructose in grams.

TABLE IX.

	Taken	Found	Left	Percentage destroyed
A.	0·4953	0·4715	0·4664	1·061
B.	0·6740	(0·647)	0·6688	0·771

The sugars used in the above experiments were: in (A) Kahlbaum's pure "laevulose cryst" and in (B) Kahlbaum's "laevulose from Inulin II," both having been dried by exposure in finely powdered layers to sulphuric acid in a vacuum desiccator for several weeks. It will be noted that in experiment (A) the percentage destruction is calculated from the fructose found, not from the sugar weighed, since the "laevulose cryst" is not so pure as "laevulose from Inulin II," and so the value for actual fructose taken as found by reduction is used, whereas in experiment (B) where the sugar was estimated by polarimetric readings, and the instrument available only read accurately to one-tenth of a degree, the percentage destruction is calculated from the actual amount of sugar weighed out.

It will also be apparent that in the case where the "laevulose cryst" is used, i.e. the less pure of the two qualities of sugar used (laevulose from Inulin II being when dried really an extremely pure sample), the loss indicated is greater than in the second case.

SUMMARY.

1. Glucose and maltose are quantitatively oxidised by bromine at room temperature when allowed to stand in a saturated solution. However the liquid still retains a small amount of its original reducing power towards alkaline copper solutions.

2. Fructose is not oxidised at all, or only to a very small extent, by bromine under these conditions.

3. In the presence of deci-normal sulphuric acid there is a slight loss of fructose in 66 hours at room temperature.

4. A mixture of sucrose, maltose, glucose and fructose may be analysed as follows:

(*a*) The amount of sucrose is determined by polarisation and reduction before and after treatment with invertase.

(*b*) The resulting mixture of reducing sugars is treated with bromine under standard conditions of acidity and temperature for a given time. The aldehydic sugars, glucose (including the portion derived from sucrose) and maltose are oxidised. The ketonic sugar, fructose (including the portion derived from sucrose), remains behind. The reducing power of the solution may then be taken as due to the fructose only.

(*c*) The rotations due to the fructose and sucrose are then allowed for in the initially determined rotation. This gives the rotation due to glucose and maltose.

(*d*) The reduction due to fructose is subtracted from the initial reduction. This gives the reduction due to glucose and maltose. From these data two equations can be constructed which when solved by the usual algebraical method give the amounts of the two remaining sugars. When only glucose and fructose are present the bromine method enables the latter to be directly determined, instead of indirectly by solving an equation.

5. The addition of dilute alkali to a solution of glucose, fructose or

maltose produces a large temporary increase in its reducing power. Consequently in analytical work sugar solutions must be kept neutral or very faintly acid.

6. From a study of the electrical conductivity of a mixture of sodium hydroxide and glucose some indication has been obtained of the formation of a compound between these two substances.

7. An unsuccessful attempt was made to modify Barfoed's solution for quantitative work, by reducing the reaction to the first order instead of the fifth.

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XLI. THE DISTRIBUTION OF ESTERASES IN THE ANIMAL BODY.

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It is to the organs more directly concerned with digestion that one naturally looks for fat-splitting ferments. Yet, as a matter of fact, the body appears to be permeated with ferments which are entrusted with the delicate mechanism of equilibrium for which their peculiar reversible action is fitted, and upon which nutrition, metabolism, and life itself depend. It may be boldly presumed that wherever any constituent of an organ is found in a constant proportion, a ferment will be found preserving that proportion. A well-known illustration of this relation may be seen in the action of the glycogen-sugar ferment of the liver and blood. Going upon this assumption, one would expect to find lipase in organs containing glycerol esters, lecithase in organs containing lecithin, wax-splitting ferments in organs containing higher alcohol esters. On the other hand, it does not follow that the presence of ferments indicates also the presence of their substrates; the ferment may be elaborated, as in the case of pancreatic steapsin, for use in another sphere of action than the site of its origin.

Fat-splitting ferments have been described in many different tissues by a number of authors, among others Loewenhart [1902] and Rona [1911, 1, 2]. Other esterases such as cholesteryl esterases and wax-splitting ferments, that is ferments which are able to split the esters of higher alcohols, also occur, as I have recorded in a former paper.

A parallel has been drawn between lypolytic power and resistance to tuberculosis. Winternitz and Meloy [1910] who have described lipase (decomposing ethyl butyrate) in a number of human organs, find a diminution of this ferment in the kidneys, liver, lungs and blood in tuberculosis. Achard and Clerc [1902, 1904] have also noticed a diminution in Hanriot's serum lipase in tuberculous conditions which were not improving.

In order to investigate the distribution of different esterases in the animal body, and incidentally to compare lipolytic activity with the known divergence as regards resistance to tuberculosis of certain species and organs, extracts were made of the following organs and examined:

	Human	Ox	Sheep	Pig	Cat	Rabbit	Guineapig
Pancreas	+	+	+	+			
Liver	+	+	+	+	+	+	+
Lung	+	+	+	+	+	+	+
Spleen	+	+	+	+	+	+	+
Kidney	+	+	+	+	+	+	+
Brain	+	+	+	+			
Suprarenals	+	+	+	+			
Thyroid	+	+	+	+			
Thymus		+	+	+			
Lymphatic glands	+	+	+	+			
Haemolymph glands		+					
Pituitaries		+	+				
Bonemarrow		+					
Salivary glands				+			
Skin	+						

In some cases, especially with human organs, which were more or less pathological, and usually less active than the normal organs obtained from the other species, material from more than one individual was examined. In the case of human lymphatic glands seven samples of tuberculous glands in addition to non-tuberculous were under investigation.

The best medium known for extracting lipase from tissues is glycerol. Lipase is soluble in pure glycerol, as also are salts. Fatty acids, alcohols and esters are only very slightly soluble, but enough to aid lipolysis and also the titration of these usually difficult soluble substances. Glycerol also preserves the lipase by abstracting water, which injures the ferment. Glycerol is indeed well known as a preservative and anti-putrefactive, being used in commerce, for example in rennet preparations, etc., for that purpose. Although ferment solutions extracted from organs are usually difficult to preserve from putrefaction, the glycerol extracts made from organs in this investigation all became sterile within a few days. That this did not depend on the direct action of the glycerol on the organisms, but rather on its preservative influence on bacteriolytic substances present, was proved by the varying rate at which the organs became sterile. Liver became sterile at once; lung was perhaps the slowest of all. This sterility allowed of certainty in ascribing lipolytic action to the organ from which the extract was made.

The method of extraction adopted was that recommended by Kanitz [1905], and is very simple. Glands were minced down, and pure glycerol added, in the proportion of one-third gland and two-thirds glycerol. After two days in contact, the extract was filtered through gauze, as lipases are easily injured by filtration. A small amount of fat may come through mechanically, but this is of no consequence if the autolytic activity of the extract itself is always measured in the presence of activating calcium chloride.

The following esters were used:

(1) Glycerol esters: butyrin, tripalmitin, tristearin, olive oil, castor oil, cod liver oil, and lecithin.

(2) Cholesterol esters: lanolin, cholesteryl propionate, cholesteryl palmitate, cholesteryl stearate (made according to Mair's [1914] directions).

(3) Beeswax, containing myricyl palmitate.

(4) Spermaceti, containing cetyl palmitate.

(5) Chinese wax, containing ceryl cerotate.

(6) Carnauba wax, containing myricyl cerotate.

The glycerol esters, with the exception of butyrin, were made up in 20 % solutions or emulsions. Butyrin was used undiluted, water being added in proportionate amount. Olive, castor, and cod liver oil were neutralised and diluted to approximately 20 %, 10 % of alcohol being present. Ovolecithin, obtained from Merck, was dissolved to 20 % in cold alcohol. Its acidity was deducted. Tristearin, tripalmitin, and the higher alcohol esters were dissolved in 40 % benzene, or xylene, and were warmed before use to allow of measurement, these substances being very insoluble. They were either neutralised in boiling methyl alcohol with methyl alcoholic potash, the alcohol being removed by evaporation, or, more often, as this first method appeared somewhat to increase insolubility, they were dissolved and used in an acid state, the initial acidity being deducted. It should be mentioned that when the fats were first obtained they were boiled in order to destroy any ferment present, as commercial preparations of fats are not always free from traces of lipases. Lecithin, although boiled, decomposes slowly in the presence of water. This process is, however, much hastened by the presence of gland extract, which contains lecithase.

The gland extracts added to fat alone, are quite inactive and require an activating agent. Activation is believed to depend upon a removal of the inhibitory products of ferment action. Thus $CaCl_2$, recommended by Kanitz [1905], has been shewn by Pekelharing [1912] to act by forming insoluble soaps of calcium, and so by precipitation, to remove the products which are hindering further action. The action of bile salts is quite opposite to this, by promoting solubility, especially in the duodenum, they promote the removal of the end products of lipolysis by their absorption through the membrane.

In testing for esterases, in each tube were placed 1 cc. of ester solution, 1 cc. of glycerol gland extract, 0·5 cc. of 5 % $CaCl_2$, and 0·5 cc. of alcohol. Controls were made containing 1 cc. of gland extract, with a similar amount of benzene, alcohol, and $CaCl_2$, but no ester. Esters were also tested alone with $CaCl_2$ and alcohol, in order to deduct their end acidity from the total acidity found after lipolysis. The mixtures were kept at room temperature and were neutralised with N/10 NaOH repeatedly during 24 hours. It is difficult to set a time limit, as, especially in the case of glycerides emulsified in water, neutralisation often restarts ferment action which had ceased, yet some mixtures act as quickly as they can be neutralised, while others may be exceedingly slow, but are never quite finished. This was especially the case with butyrin. Most mixtures could not be carried beyond a certain definite limit, which was quickly reached. This was especially the case with the higher alcohol esters and was no doubt largely due to the fact that in benzene they were mainly in a dissolved condition, which certainly aids hydrolysis.

RESULTS.

In the different glands examined fat- and wax-splitting ferments were found, which appeared to be quite independent of each other. The gland extracts differed greatly in lipolytic properties, both in strength and kind. Butyrinase was always present, although varying greatly in strength. As lecithin is so essential a constituent of cells, it is not surprising that lecithinase should appear quite as constantly. Lecithin is, however, decomposed by water and $CaCl_2$; the presence of lecithase is only determined by increased rate of decomposition so that the demonstration of small quantities of the ferment is not very satisfactory. Action on unsaturated glycerides such as olein was also rather commonly found, but often very slight. Many organs had no effect on tristearin; those which had the most influence on this rather insoluble fat were also able to deal with the higher alcohol esters, suggesting, once again, a connection between solubility and lipolytic power. Ferments hydrolysing the esters of the higher alcohols were found in one sample of human skin in quite remarkable strength. In pancreas extract they were also strong, and were present in a lesser degree in the liver, thymus, suprarenal, lymphatic, and other glands. On comparing the lipolytic power of the different species examined, some interesting facts are apparent. The pig has been regarded as possessing more lipolytic power than the other species, but this superiority is practically entirely limited to the pancreas. Lipases are widely distributed in the organs of the pig, but in no greater measure than in the organs of other animals. Certain organs of the sheep, especially pancreas, liver and spleen, are considerably less lipolytic than in the pig, so that these organs certainly afford no explanation of the greater susceptibility of the pig to tuberculosis. If, however, the lymphatic glands of these two species are compared, a very different relationship will be found. While sheeps' lymphatic glands were found remarkably active, being the most active lymphatic gland extracts examined, pigs' glands were decidedly the weakest. The pig is susceptible to glandular tuberculosis. As ingested tubercle is believed to enter the body by filtration through lymphoid tissue (tonsils and ileo-caecal region), and then via the lymphatic glands, this lipolytic activity in the sheeps' lymphatic glands may well explain the resistance of the sheep to this disease. With the exception of one sample of human skin, human organ extracts were found disappointingly weak on the whole. On account of their source, and more or less pathological condition, they cannot be fairly compared with normal organs from other species. Nevertheless, although the results given by them are not striking, they indicate a widespread, decided and fairly even lipolytic activity over the very different varieties of fats used. Except for lecithase in the brain, the only superiority which human glands displayed was seen in a kidney shewing fatty degeneration, where there was unusual lipolytic activity, especially in the form of butyrinase. The guineapig, which is so susceptible to tuberculosis, proved decidedly weak in lipolytic power, except with regard to butyrin. The cat, on the other hand, resistant to tuberculosis, yielded stronger ferments, more especially against the higher alcohol esters. It is interesting that the brain, which contains cholesterol, but no cholesteryl ester [Lorrain Smith and Mair, 1912], possesses no cholesteryl esterases. On the other hand, those organs which are known to contain cholesteryl esters, *i.e.*, skin and suprarenals, contain also higher alcohol esterases.

In the following tables, shewing esterase activity, any primary fat acidity has been deducted, but not gland acidity. The acidity of the gland alone is, however, always given below. The gland acidity, and low or negative esterase figures close to it, are printed in italics, so that positive results are more conspicuous. The numbers throughout represent cc. of N/10 NaOH required to neutralise the mixtures within 24 hours.

ESTERASE ACTIVITY.

Pancreas.

	Substrate	Maximum acidity*	Human	Ox	Sheep	Pig
1.	Butyrin	19·9	11·7	16·0	16·0	16·0
2.	Palmitin	7·2	*0·6*	1·0	—	5·6
3.	Stearin	6·7	*0·4*	0·7	0·75	5·6
4.	Cod-liver oil	6·7	3·6	1·9	1·75	5·0
5.	Castor oil	6·6	3·0	2·0	2·0	4·4
6.	Olive oil	7·0	4·4	2·7	2·0	5·0
7.	Lecithin	—	—	7·0	7·0	7·0
8.	Lanolin	7·0	7·0	1·0	1·6	5·1
9.	Beeswax	6·7	*0·4*	0·9	1·1	5·1
10.	Cholesteryl stearate ...	6·2	*0·7*	1·0	1·0	3·0
11.	Cholesteryl palmitate ...	6·4	*0·3*	1·0	1·0	3·5
12.	Cholesteryl propionate ...	9·0	*0·4*	1·6	1·2	5·0
13.	Carnauba wax	6·6–6·8	*0·4*	0·6	*0·6*	3·2
14.	Chinese wax	—	—	0·6	*0·6*	3·3
15.	Spermaceti	8·8–9·6	—	0·8	1·25	4·5
	Gland control	—	*0·3*	*0·2*	*0·35*	*0·9*

* The maximum acidity is calculated from the saponification value.

Lung.

	Substrate	Human	Ox	Sheep	Pig I	Pig II	Cat	Rabbit	Guinea-pig
1.	Butyrin	1·0	0·6	1·8	—	0·4	2·2	*0·5*	1·2
2.	Palmitin	*0·3*	*0·15*	*0·1*	—	*0·1*	*0·35*	*0·3*	*0·2*
3.	Stearin	*0·3*	*0·15*	*0·35*	—	*0·1*	*0·35*	*0·3*	*0·2*
4.	Cod-liver oil ...	*0·3*	*0·1*	*0·25*	—	*0·2*	*0·35*	*0·3*	*0·2*
5.	Castor oil	*0·3*	*0·15*	*0·3*	—	*0·2*	*0·35*	*0·2*	*0·2*
6.	Olive oil	*0·4*	*0·2*	0·6	0·8	*0·2*	0·45	*0·3*	*0·2*
7.	Lecithin	*0·7*	1·8	1·9	1·4	1·5	1·5	*0·6*	*0·7*
8.	Lanolin	*0·5*	*0·15*	*0·3*	—	*0·2*	*0·25*	*0·2*	*0·15*
9.	Beeswax	*0·3*	*0·15*	*0·45*	—	*0·2*	*0·2*	*0·3*	*0·2*
10.	Cholesteryl stearate	*0·4*	—	—	—	—	*0·2*	*0·3*	*0·2*
11.	Cholesteryl palmitate	*0·4*	—	—	—	—	*0·3*	*0·3*	*0·2*
12.	Cholesteryl propionate	*0·5*	—	—	—	—	*0·3*	*0·3*	*0·2*
13.	Carnauba wax ...	*0·3*	—	—	—	—	*0·2*	—	—
14.	Chinese wax ...	*0·3*	—	—	—	—	*0·25*	—	—
15.	Spermaceti	*0·6*	—	—	—	—	*0·4*	—	—
	Gland control ...	*0·4*	*0·15*	*0·3*	*0·2*	*0·2*	*0·25*	*0·4*	*0·2*

Lymphatic glands.

	Substrate	Human		Ox	Sheep	Pig
		Tub.	Normal			
1.	Butyrin	0·6	0·7	2·2	12·5	1·0
2.	Palmitin	*0·4*	*0·15*	1·0	1·0	*0·2*
3.	Stearin	*0·3*	*0·15*	1·0	1·0	*0·25*
4.	Cod-liver oil	*0·25*	*0·15*	1·1	3·5	0·5
5.	Castor oil	*0·3*	*0·15*	1·1	3·7	*0·3*
6.	Olive oil	*0·35*	*0·15*	1·1	3·5	0·5
7.	Lecithin	1·4	0·8	2·25	2·5	1·0
8.	Lanolin	*0·35*	*0·15*	0·9	0·8	*0·25*
9.	Beeswax	*0·35*	*0·15*	1·2	1·4	*0·25*
10.	Cholesteryl stearate	*0·25*	*0·15*	1·0	1·0	*0·35*
11.	Cholesteryl palmitate	*0·4*	*0·15*	1·0	1·0	*0·3*
12.	Cholesteryl propionate	*0·4*	*0·15*	0·8	*0·7*	*0·3*
13.	Carnauba wax	*0·35*	*0·15*	—	—	*0·2*
14.	Chinese wax	*0·35*	*0·15*	—	—	*0·25*
15.	Spermaceti	*0·45*	*0·25*	—	—	*0·4*
	Gland control	*0·25*	*0·15*	*0·4*	*0·5*	*0·2*

Brain.

	Substrate	Human	Ox	Sheep	Pig
1.	Butyrin	1·2	0·55	0·7	0·7
2.	Palmitin	*0·2*	*0·2*	*0·4*	*0·2*
3.	Stearin	*0·2*	*0·2*	*0·4*	*0·2*
4.	Cod-liver oil	*0·3*	*0·2*	*0·4*	*0·3*
5.	Castor oil	*0·3*	*0·25*	*0·4*	*0·3*
6.	Olive oil	*0·3*	*0·3*	*0·4*	*0·3*
7.	Lecithin	7·0	2·3	2·3	2·0
8.	Lanolin	*0·2*	*0·2*	*0·4*	*0·15*
9.	Beeswax	*0·25*	*0·2*	*0·5*	*0·2*
10.	Cholesteryl stearate	*0·2*	*0·2*	0·9	*0·2*
11.	Cholesteryl palmitate	*0·2*	*0·2*	*0·4*	*0·2*
12.	Cholesteryl propionate	*0·3*	*0·2*	*0·4*	*0·2*
13.	Carnauba wax	*0·2*	—	—	—
14.	Chinese wax	*0·2*	—	—	—
15.	Spermaceti	*0·3*	—	—	—
	Gland control	*0·2*	*0·2*	*0·4*	*0·2*

Haemolymph glands.

	Substrate	Ox
1.	Butyrin	1·2
2.	Palmitin	*0·5*
3.	Stearin	*0·5*
4.	Cod-liver oil	*0·45*
5.	Castor oil	*0·45*
6.	Olive oil	0·6
7.	Lecithin	1·3
8.	Lanolin	*0·5*
9.	Beeswax	*0·5*
10.	Cholesteryl stearate	*0·5*
11.	Cholesteryl palmitate	*0·4*
12.	Cholesteryl propionate	*0·4*
13.	Carnauba wax	0·8
14.	Chinese wax	*0·5*
15.	Spermaceti	*0·5*
	Gland control	*0·35*

Suprarenals.

	Substrate	Human	Ox	Sheep	Pig I	Pig II
1.	Butyrin	*0·65*	1·7	1·9	1·25	1·4
2.	Palmitin	*0·65*	1·1	*0·65*	*0·6*	0·75
3.	Stearin	*0·65*	1·1	*0·6*	*0·6*	*0·65*
4.	Cod-liver oil	*0·65*	1·0	*0·6*	*0·6*	*0·55*
5.	Castor oil	*0·65*	*0·7*	*0·7*	*0·55*	*0·6*
6.	Olive oil	*0·65*	1·0	*0·7*	*0·6*	*0·6*
7.	Lecithin	1·45	1·8	1·3	1·5	1·75
8.	Lanolin	*0·75*	1·0	*0·6*	0·65	*0·7*
9.	Beeswax	*0·75*	1·5	*0·55*	*0·55*	*0·5*
10.	Cholesteryl stearate	*0·7*	1·1	*0·45*	*0·4*	*0·6*
11.	Cholesteryl palmitate	*0·7*	1·1	*0·55*	*0·35*	*0·6*
12.	Cholesteryl propionate	*0·7*	1·1	*0·55*	*0·4*	*0·6*
13.	Carnauba wax	*0·75*	—	*0·55*	*0·55*	*0·65*
14.	Chinese wax	*0·8*	—	*0·65*	*0·55*	*0·65*
15.	Spermaceti	1·0	—	*0·65*	0·75	0·75
	Gland control	*0·65*	*0·6*	*0·5*	*0·35*	*0·45*

Thymus.

	Substrate	Ox	Sheep	Pig
1.	Butyrin	3·6	5·1	2·1
2.	Palmitin	1·2	1·0	1·4
3.	Stearin	1·0	1·0	1·0
4.	Cod-liver oil	1·4	1·1	1·1
5.	Castor oil	1·4	1·1	0·9
6.	Olive oil	1·7	1·2	1·1
7.	Lecithin	2·7	2·7	3·0
8.	Lanolin	2·0	1·1	1·0
9.	Beeswax	1·7	1·3	1·0
10.	Cholesteryl stearate	1·8	1·2	0·9
11.	Cholesteryl palmitate	1·4	1·2	0·8
12.	Cholesteryl propionate	1·0	1·2	1·0
13.	Carnauba wax	0·9	0·9	0·8
14.	Chinese wax	0·8	1·0	0·8
15.	Spermaceti	1·5	1·0	0·8
	Gland control	*0·3*	*0·5*	*0·5*

Liver.

	Substrate	Maximum acidity	Human	Ox	Sheep	Pig	Cat	Rabbit	Guinea-pig
1.	Butyrin	19·9	4·0	12·4	8·5	16·0	14·0	16·0	16·0
2.	Palmitin	7·2	2·0	1·6	*0·2*	*0·5*	1·3	2·0	0·9
3.	Stearin	6·2	1·8	1·6	*0·3*	*0·4*	1·0	2·0	*0·4*
4.	Cod-liver oil	6·7	2·0	2·8	*0·4*	0·8	1·0	2·0	1·15
5.	Castor oil	6·6	2·0	2·9	*0·4*	1·0	1·0	2·0	1·0
6.	Olive oil	7·0	2·0	2·9	*0·5*	1·0	1·0	*1·7*	1·9
7.	Lecithin	—	6·9	5·5	5·5	6·8	4·8	6·0	3·3
8.	Lanolin	7·0	*1·2*	2·4	*0·4*	1·4	1·2	2·0	*0·4*
9.	Beeswax	6·7	2·1	2·0	*0·3*	1·2	*0·9*	2·0	*0·8*
10.	Cholesteryl stearate	6·2	1·8	2·4	*0·3*	1·2	1·1	2·0	*0·5*
11.	Cholesteryl palmitate	9·0	1·8	2·2	*0·4*	1·0	*0·8*	2·0	*0·5*
12.	Cholesteryl propionate	9·0	1·8	2·4	*0·5*	1·2	1·0	2·0	*0·5*
13.	Carnauba wax	5·6–6·8	*1·2*	1·4	*0·4*	0·8	*0·8*	—	*0·5*
14.	Chinese wax	—	*1·2*	1·4	*0·3*	0·7	1·1	—	*0·5*
15.	Spermaceti	8·8–9·6	*1·4*	2·6	*0·5*	1·2	1·3	—	*0·6*
	Gland control	—	*1·3*	*0·3*	*0·3*	*0·4*	*0·6*	*1·5*	*0·5*

Spleen.

	Substrate	Human	Ox	Sheep	Pig	Cat
1.	Butyrin	1·0	0·8	0·5	1·8	1·5
2.	Palmitin	*0·3*	*0·6*	*0·3*	*0·8*	*0·8*
3.	Stearin	*0·25*	*0·6*	*0·3*	*0·75*	*0·8*
4.	Cod-liver oil	*0·35*	*0·5*	*0·3*	1·25	*0·8*
5.	Castor oil	*0·35*	*0·4*	*0·3*	1·25	*0·8*
6.	Olive oil	*0·35*	0·7	*0·5*	1·7	*0·8*
7.	Lecithin	4·3	2·4	2·3	7·0	5·0
8.	Lanolin	*0·3*	*0·4*	*0·4*	*0·6*	*0·7*
9.	Beeswax	*0·3*	0·8	0·7	*0·8*	*0·8*
10.	Cholesteryl stearate	*0·3*	*0·4*	*0·3*	*0·7*	*0·6*
11.	Cholesteryl palmitate	*0·3*	*0·4*	*0·3*	*0·7*	*0·6*
12.	Cholesteryl propionate	*0·3*	*0·4*	*0·4*	*0·7*	*0·6*
13.	Carnauba wax	*0·3*	—	—	—	*0·5*
14.	Chinese wax	*0·3*	—	—	—	*0·6*
15.	Spermaceti	*0·3*	—	—	—	0·9
	Gland control	*0·3*	*0·4*	*0·3*	*0·7*	*0·6*

Kidney.

	Substrate	Human	Ox	Sheep	Pig	Cat
1.	Butyrin	(fatty) 6·65	0·8	0·9	—	4·0
2.	Palmitin	2·2	*0·3*	*0·4*	—	0·65
3.	Stearin	2·2	*0·3*	*0·4*	—	0·55
4.	Cod-liver oil	2·5	*0·4*	*0·4*	—	0·6
5.	Castor oil	2·4	*0·4*	*0·4*	—	0·6
6.	Olive oil	*1·8*	*0·4*	*0·4*	—	0·7
7.	Lecithin	2·5	1·05	1·2	—	1·35
8.	Lanolin	2·2	*0·4*	*0·4*	—	*0·4*
9.	Beeswax	*2·0*	0·6	*0·5*	—	*0·45*
10.	Cholesteryl stearate ...	3·0	*0·3*	*0·4*	—	*0·45*
11.	Cholesteryl palmitate ...	2·4	*0·3*	*0·4*	—	*0·45*
12.	Cholesteryl propionate ...	2·2	*0·4*	*0·4*	—	*0·45*
13.	Carnauba wax	—	—	—	—	*0·35*
14.	Chinese wax	—	—	—	—	0·55
15.	Spermaceti	—	—	—	—	0·6
	Gland control	*1·8*	*0·3*	*0·4*	—	*0·25*

Thyroid.

	Substrate	Ox	Sheep	Pig
1.	Butyrin	1·6	4·3	0·4
2.	Stearin	0·9	*0·1*	*0·1*
3.	Cod-liver oil	0·7	0·4	*0·15*
4.	Castor oil	0·8	*0·2*	*0·1*
5.	Olive oil	0·8	*0·3*	*0·1*
6.	Lecithin	3·3	2·3	0·85
7.	Lanolin	1·0	0·4	*0·1*
8.	Beeswax	0·9	0·4	*0·1*
	Gland control ...	*0·4*	*0·1*	*0·1*

Skins. Bile. Salivary glands.

	Substrate	Skins, human			Bile, Ox	Salivary glands, Pig
		1	2	3		
1.	Butyrin	6·5	2·1	—	3·5	*0·55*
2.	Palmitin	4·2	0·7	—	*0·5*	*0·25*
3.	Stearin	4·0	1·1	—	*0·5*	*0·2*
4.	Cod-liver oil	3·5	0·65	—	*0·5*	*0·25*
5.	Castor oil	3·0	0·65	—	*0·5*	*0·3*
6.	Olive oil	*0·2*	0·65	—	*0·8*	*0·3*
7.	Lecithin	1·6	1·0	—	*0·5*	1·3
8.	Lanolin	3·8	0·9	0·35	*0·5*	*0·2*
9.	Beeswax	4·5	1·0	0·35	*0·5*	*0·2*
10.	Cholesteryl stearate ...	4·7	1·0	0·4	*0·5*	*0·2*
11.	Cholesteryl palmitate ...	5·0	0·85	0·35	*0·5*	*0·25*
12.	Cholesteryl propionate ...	4·2	0·75	0·35	—	*0·2*
13.	Carnauba wax	—	0·75	0·45	—	*0·2*
14.	Chinese wax	—	0·7	0·4	—	*0·2*
15.	Spermaceti	—	0·75	0·45	—	*0·25*
	Tissue control	*0·2*	*0·15*	*0·05*	*0·5*	*0·2*

	Substrate	Pituitary.		Bone marrow.
		Ox	Sheep	Ox
1.	Butyrin	0·8	0·4	3·4
2.	Palmitin	0·65	0·1	0·8
3.	Stearin	0·55	0·1	0·8
4.	Cod-liver oil	0·75	0·1	0·8
5.	Castor oil	0·5	0·1	0·7
6.	Olive oil	0·7	0·1	0·7
7.	Lecithin	1·2	0·85	1·8
8.	Lanolin	0·5	0·2	0·7
9.	Beeswax	0·55	0·15	0·8
10.	Cholesteryl stearate...	0·45	0·2	—
11.	Cholesteryl palmitate	0·55	0·25	—
12.	Cholesteryl propionate	0·5	0·25	—
13.	Carnauba wax ...	0·5	0·1	—
14.	Chinese wax	0·65	0·1	—
15.	Spermaceti	0·5	0·15	—
	Gland control ...	0·3	0·1	0·6

CONCLUSIONS.

Fat- and wax-splitting ferments are widely distributed among the organs of man, ox, sheep, pig, cat, rabbit, and guineapig.

Butyrinase and lecithase were present in all of the 58 organs examined, and were often very strong.

Olein lipase was also commonly present, but often as a mere trace. It was strongest in pigs' pancreas, but also active in the pancreas of other species, in the liver, and in the thymus, and to a lesser degree in lymphatic glands, ox suprarenals and thyroid, cat kidney, and pig lung.

Tristearin ferment was less frequently found, but was active in the ox, sheep and pig pancreas, the human, ox, cat and rabbit liver, the thymus, ox and sheep lymphatic glands, the human skin, human and cat kidney, ox suprarenals, and ox thyroid.

Wax-splitting ferments were found in a variety of organs, ox, sheep and pig pancreas, human, ox, pig, cat and rabbit liver, thymus, lymphatic glands, ox and sheep thyroid, ox suprarenals, human skin, and cat and human kidney.

The distribution of esterases in the body appears to have some relation to resistance to tubercle. The cat, which is very little susceptible to tubercle, is well provided with esterases throughout its organs. The sheep, although less well provided for than the susceptible pig in other regions, possesses very active lymphatic glands. The susceptible guineapig has the chief part of its lipase in the form of butyrinase and olein-lipase, and is deficient in higher esterases. The lungs in all species were found very poor in these ferments. The more resistant liver shewed active lipolytic properties.

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XLII. THE GROWTH OF RATS UPON DIETS OF ISOLATED FOOD SUBSTANCES.

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(*Received October 23rd, 1916.*)

In a recent communication on the growth of rats upon artificial diets containing lactose, published in this journal, Drummond [1916] has discussed a number of problems with which we, as well as other investigators, have been concerned for several years. The facts reported are essentially in harmony with our own experience in the study of the growth of white rats fed upon mixtures of isolated food substances. Inasmuch as readers of Drummond's paper who have not carefully followed our own publications might easily gain the impression that our views are at variance with his conclusions as well as with those of some other workers in this field at the present time, we regard it as desirable to refer here to a few of the topics involved.

In order to appreciate some of the changes of opinion which have taken place as the result of more recent studies in feeding animals on isolated food mixtures, it should be pointed out that earlier we have recorded experiments showing successful growth for such considerable periods that it seemed reasonable, at that time, to conclude that the diets employed were fully adequate for prolonged nutrition. Subsequent and more extended experience demonstrated, however, that failure and decline of body weight inevitably ensued unless additional accessory substances were furnished in the diet. By the use of what we termed "protein-free milk," along with isolated proteins, starch, and fats in the form of lard, we usually succeeded in promoting the growth of albino rats for about eighty days; and when the failure on these diets manifested itself, the decline was averted by the substitution of certain natural fats, notably butter fat, for a part of the lard. This agrees with the experience of other investigators in this field. The inevitable ultimate failure of all experiments in which a fat-soluble accessory component was missing was at first overlooked, owing to the fact that the earlier observations were made over too short a period of time to permit the nutritive decline to manifest itself. This statement applies to the results of the feeding experiments with fat-free food mixtures which we published in 1912 [Osborne and Mendel 1912]. We have not claimed, however, that the diets in these experiments were "*lipoid*"-free. We considered them as evidence of the possibility of very considerable constructive nutrition in the absence of the ordinary fats. Indeed, some time ago we attempted to avoid any misconception regarding our previous experience by the following statement: "In reviewing our earlier published experiments Funk has erroneously assumed that we secured completed growth with the diets in which the butter component was not yet employed. It is true that the increments in weight were in some cases very noteworthy; but in every instance cessation ultimately ensued before the completion of the normal progress of the growth or subsequent maintenance. We have never denied the necessity of a growth-promoting food accessory in accord with the claim of Hopkins; and recently we pointed out that the successful partial completion of growth, such as has been obtained in our experiments, may well have been due to a store of the essential compound in the body of the experimental animals at the beginning of the trials. It is by no means necessary to assume with Funk that small quantities of these accessory substances were inadvertently left in our food preparations owing to insufficient extraction with alcohol" [1913, 2, p. 430].

After our discovery that "protein-free milk" together with starch, butter fat, and an adequate protein furnishes a suitable ration for rats, we imitated the inorganic ingredients of the "protein-free milk" by a suitable combination of bases and acids,—our "artificial protein-free milk." When this was used in place of the natural product we obtained considerable growth in several instances; but in the great majority of cases little, if any, growth was made. McCollum and Davis [1915] also have reported not a few examples of very considerable growth upon similar rations consisting of isolated food substances and artificial salt mixtures. The possibility of obtaining adequate growth in this way is very slight; and it is always merely a question of time before a nutritive decline ensues unless a change is instituted in the ration, whereby the lacking accessory substances are furnished. Whether the growth occasionally made on these purely artificial mixtures is caused by impurities attached to the lactose used—as has been intimated by Drummond—or whether certain animals are possessed of a sufficient store of nutrition-promoting "hormones" to enable them to utilise highly purified isolated food substances in a constructive way for some time, is debatable in view of the very small proportion of rats which make any material growth of such foods.

Our attitude was summarised in 1913 as follows: "Our own experiments, as well as those of the other investigators mentioned, make it clear that something further than a sufficient supply of energy-yielding food material is required to promote a normal growth. The animal cells need for their activities not only energy, but also suitable constructive material to replace the wear and tear therein. Furthermore the cells are concerned in the elaboration of a great diversity of complex and little understood substances such as enzymes, products of internal secretion, etc., which unquestionably play an indispensable rôle in life and may require either special antecedent products for their construction, chemical activators of some sort, or minute quantities of readily overlooked rarer elements and compounds. It is easy, yet futile at the present time, to develop detailed hypotheses respecting the almost innumerable possibilities involved. The greatest promise of success in discovering the food factors which determine successful growth lies in seeking them in some chemical constituents of such diets as have proved adequate to promote growth.

"In attempting to ascertain what constitutes an adequate diet, by feeding experiments with isolated substances, we have found that our purely artificial foods—mixtures of isolated proteins, fats, carbohydrates and inorganic salts—sooner or later fail to maintain mature animals. In view of this such dietaries may naturally be expected to fail to maintain the less resistant young during their adolescent period. It is true that in several instances we have succeeded in keeping grown rats in health and in apparent nutritive equilibrium on purely artificial food mixtures over periods far longer than the experience of our predecessors had led us to expect. But the outcome has never been satisfactory in the sense of extending over what may be considered as the larger portion of the life span of an adult animal. Successful maintenance has been secured only when the animals were fed, in part at least, with foods containing our 'protein-free milk,' the preparation and composition of which has been detailed elsewhere. The superiority of the latter foods, compared with any purely artificial food mixture in repairing the depleted body weight of animals that have begun to decline on the artificial salt mixtures tested, is beyond question. Instances of immediate recovery following the replacement of the inorganic constituents of the dietaries and part of the carbohydrates by the 'protein-free milk' have been published, and might be duplicated in great numbers from our protocols. Even greater success is manifested in maintenance experiments in which the 'protein-free milk' alone furnished the inorganic constituents of the dietary during long periods of time. The superiority of the 'protein-free milk' foods over the diets containing artificial salt mixtures in the maintenance experiments also is unquestioned. Wherein the difference lies is not yet apparent. We have already pointed out that the efficiency of this adjuvant to the energy-yielding nutrients is not attributable to the minute trace of milk protein present. The fact of the greater efficiency of the natural milk product suggests that some constituent present in milk is essential for prolonged maintenance" [1913, 1, p. 312].

As a result of the circumstances here recorded we were led, like McCollum and Davis, to investigate the fat-soluble components (McCollum's fat-soluble A) that promote growth in the presence of other suitable dietary ingredients.

Similarly one of us has recently stated [Mendel 1915]: "It is not unlikely —to speak conservatively—that there are at least two 'determinants' in the nutrition of growth. One of these is furnished in our 'protein-free milk' which insures proper maintenance even in the absence of growth. When this was fed we have maintained rats without growth for very long periods. Without this 'determinant' (as, for example, in diets of isolated food substances containing artificial substitutes for 'natural' protein-free milk) the special components of butter fat or cod-liver oil or egg fat induce only limited gains at best. Another 'determinant' is furnished by these natural fats (or fractions more recently prepared therefrom by Osborne and myself or the saponification product of McCollum and Davis). Either of the determinants may become 'curative'; both are essential for growth when the body's store of them (if such there be) becomes depleted. It is too early to attempt a tenable conclusion."

We believe the foregoing statements published by us some time ago should make it apparent that, like Drummond, we have concluded "before complete growth can occur in a young animal, the diet, besides being adequate as regards its proteins, carbohydrates, fats and salts, must contain both fat-soluble and water-soluble accessory substances" [Drummond 1916, p. 102].

Drummond has stated [1916, p. 93]: "Osborne and Mendel have always been careful to utilise, throughout their extensive researches, proteins of a very high degree of purity, and yet they have gone to the other extreme in continuing to use in their diets 'protein-free milk.' Reference has already been made to the criticisms which have been directed against the use of this complex substance, as a source of lactose and salts." In this connection the reader should be reminded that our primary object was the study of the relative nutritive value of certain proteins which can be undertaken only when *all* the other (non-protein) essentials of the diet are available. The use of "protein-free milk," to which exception has been made above, has proved valuable in that it has offered an unusually good source for the water-soluble "accessory," "vitamine," "determinant," or food "hormone" which, along with suitable fat-soluble accessory, has enabled us to conduct experiments, which otherwise would have been impossible, on the relative value of certain proteins and amino-acids[1]. For our *studies of the rôle of proteins in nutrition* it has been essential that all necessary food "hormones" should be included in the diet. Consequently if the lactose used actually contained traces of "vitamines," this would offer no disadvantage for the conduct of such experiments. Obviously this does not apply to the use of such a product for investigations of the nature of the food hormones or vitamines.

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[1] The possible significance of the small quantity of protein present in our preparation of "protein-free milk" has not been overlooked. [See Osborne and Mendel, 1915.]

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XLIII. THE PECTIC SUBSTANCES OF PLANTS.

(*Preliminary communication.*)

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(*Received November 14th, 1916.*)

The substances known as the pectins, which are said to form the middle lamella of plant cells, offer many points of interest. It is supposed that they are readily attacked by various fungi which are the causes of plant diseases, and a knowledge of their chemistry is, therefore, of considerable importance to plant pathologists. They also, under certain conditions, which are not yet clearly understood, form gels, and it was mainly the interest in this particular property which first attracted the authors of this paper to their investigation. One of them (S. B. Schryver) had already commenced a systematic chemical research, when the other [Haynes 1914] published a paper dealing with the conditions under which pectic gels were formed, and they decided to continue in partnership their researches, which have, unfortunately, been repeatedly interrupted by other work.

Very little of the literature dealing with the subject of the pectins is of recent date, and the statements made by earlier authors are often confused and contradictory. It is not proposed to discuss the literature in any detail in this place, as it is dealt with in an adequate manner in various hand-books. (See more especially the works of Czapek [1913] and Tollens [1914].)

The materials employed by the authors for investigation comprised turnips, strawberries, rhubarb stem and apples, and the results so far obtained indicate that one single substance of acidic character is obtained from all sources; this has been designated *pectinogen*. On treatment with weak solutions of caustic alkalis at ordinary room temperature it readily undergoes conversion into a second substance to which the name *pectin* has been given from which gels can be readily obtained. It must be admitted that the evidence as to the identity of the pectinogen from all the sources investigated cannot be considered as complete, but as relatively large amounts of material are now available for further work, it is hoped that subsequent researches which it is proposed to carry out in this laboratory, will lead to a definite conclusion as to this point. All the samples of pectinogen, however (which has not yet been isolated in a pure state) can be converted into pectins under the same conditions; the pectins from all sources, furthermore, give on combustion almost identical analysis numbers, which correspond very closely with a definite chemical formula containing seventeen carbon atoms (or multiples thereof); the furfural obtained by the distillation with hydrochloric acid of the pectinogen from two sources has also been estimated and the results indicate very definitely that each complex of seventeen carbon atoms contains one pentose group. The preliminary results point, therefore, to the conclusion that there is a single pectin which is a complex built up by the combination of a pentose group with another group containing twelve carbon atoms. There is no evidence that it is a carbohydrate, but it appears to be an acid *sui generis*. As several hundred grams have now been prepared, an opportunity exists for a more complete chemical research on the nature of this interesting substance.

PREPARATION OF PECTINOGEN.

The principle of the method employed is the same for all materials used, but it must be varied in details to suit their different textures. The expressed juices of the plant tissues appear to contain only small amounts of pectinogen, and only in the case of apples has any appreciable amount been obtained from such sources. It is generally extracted from the plant residues which remain after the expression of the juices by treatment with warm 0·5 % ammonium oxalate solution; from this extract after concentration to a small bulk it can be precipitated in a gelatinous form by the addition of acidified alcohol. By far the largest amount of pectinogen prepared in this laboratory has been obtained from turnips (or swedes) and these give the most satisfactory yield of all the materials hitherto investigated.

Preparation from turnips. The turnips are first coarsely disintegrated in a motor-driven mincing machine and the material thus obtained is placed in a cloth and pressed as rapidly and completely as possible in an ordinary tincture press; the juice collected, which contains no appreciable amount of pectinogen, is discarded. The residue is then mixed with acetone, a procedure which tends to stop fermentative action, and also facilitates the subsequent drying processes. About 2½ litres of acetone were mixed with the residue from about 40 lbs. of turnips (one Winchester quart to one bushel of vegetables). The moistened mass was then pressed again through the mincing-machine which was "set" to the small apertures so as to obtain the material in a finer state of aggregation. It was then wrapped in a cloth and pressed, first in a tincture press and then in a Buchner press at about 200 atmospheres pressure. The residue obtained in this way was then very rapidly dried (a process which takes a relatively short time) in a current of air at 37°, a "serum-drier" supplied by Hearsons having been employed for this operation. The powder obtained in this way was then finely ground by passage through a motor-driven grinding mill.

In one experiment 4730 g. of dry material were obtained from about 100 kilos of turnips.

The powder was then washed with cold water to free it from the last traces of soluble product. The amounts of water used for washing and of ammonium oxalate solution for the subsequent extraction of the pectinogen are so chosen that a paste is formed which can be enveloped in a cloth and pressed out afterwards in a tincture press. It is not always easy to do this, for the dried powder swells very considerably on the addition of water, and different powders absorb different amounts of the liquid. If the ratio of powder to water is too high, it is difficult to express the liquid in a press; if, on the other hand, too much water is added, a paste is obtained which is too thin to manipulate in the manner described. In this latter case large amounts of liquid must be used for washing and extraction and separated from the solid by decantation. By practice, however, such a contingency can be generally avoided.

Details of the experiment already mentioned will serve to illustrate the process. The 4730 g. of powder obtained from 100 kilos of turnips were washed twice with 24 litres of water, the washings being expressed each time with the tincture press. The moist residue was then divided into two approximately equal portions, each of which was extracted twice with hot 0·5 % ammonium oxalate solution. This solution was heated to 80–90° in an enamelled bath, and the moist powder was then thrown in and well stirred with a wooden spoon; the paste thus obtained was wrapped quickly in a cloth and pressed whilst hot in a tincture press, a viscid extract being thereby obtained; the second extract of the first portion was used for the first extraction of the second portion; altogether, including the solution used for washing the cloths, 24 litres of extract were obtained. This ammonium oxalate extract was then concentrated *in vacuo* (temperature of water bath 40–45°) to about 8 litres. Chloroform and toluene were added to the liquids as soon as they were obtained, to prevent growth of moulds and bacteria which readily attack pectinogen solutions, and small amounts of these liquids were also added from time to time to the extracts during the course of their concentration. To the concentrated extract of 8 litres, about 2–3 times the volume of 95 % alcohol was added, and the pectinogen then separated in the form of a very voluminous gelatinous precipitate. This was filtered off on to blotting paper contained in large funnels, and when it had drained sufficiently it was removed from the filters, wrapped in a cloth and pressed. The cake thus obtained was then redissolved in water, a process which requires several hours and occasional vigorous stirring. The turbid aqueous solution obtained in this way was then filtered through blotting paper pulp on a large Buchner funnel. It was necessary to change the pulp several times in the course of the filtration as it soon became clogged. All the pulp used was collected and heated with water in a beaker immersed in a water-bath and the washings thus obtained were filtered off on to a Buchner funnel, the residue being well pressed and subsequently washed on the funnel. These washings were added to the filtrate, and the total bulk in this particular experiment amounted to 12 litres. The opalescent fluid thus obtained still contained small quantities of suspended particles which could be separated from the fluid by centrifugalisation. (This operation was generally omitted at this stage when large amounts of pectinogen were being prepared owing to the great bulk of the fluid. In most of the work, the crude pectinogen was purified in small quantities as required; for this purpose it was dissolved in water to yield about a 3–4 % solution which was then centrifuged; the liquid was then poured off from the deposit and the purified pectinogen was precipitated from the cleared fluid by alcohol.) The fluid was then evaporated to about 5 litres, rendered distinctly acid to Congo red by dilute hydrochloric acid, and the pectinogen was precipitated by the addition of about twice the volume of 95 % alcohol. The gelatinous precipitate was then filtered, washed on the filter with 75 % then with 95 % alcohol, pressed, and finally allowed to stand for some time under absolute alcohol. It was then filtered off and air-dried, and was thus obtained in the form of a granular white powder. Yield from 100 kilos of turnips 355 g., which on heating in a steam-oven lost nearly 25 % of its weight.

Preparation from strawberries. About 150 lbs. of strawberries, after separation of the stalks, were mashed up with about 10 litres of acetone.

The mash was filtered on to cloths, and the residue thus obtained was allowed to stand under alcohol and then pressed, first in a tincture press, then in a Buchner press; it was then dried at 37° in a current of air. The dry powder weighed about 1·5 kilos. It was washed 5 times by decantation with large quantities of water and then extracted, first with 24 litres and afterwards with 8 litres of warm 0·5 % ammonium oxalate solution. The extract was evaporated to 6 litres before precipitation with alcohol. The purification of the precipitate was carried out by the method described in the case of pectinogen from turnips. 150 lbs. yielded about 95 g. of air-dried product.

Preparation from apples. The apples were minced and pressed in a tincture press, the juice was concentrated to a small bulk and alcohol was added. The gelatinous precipitate thus obtained was filtered off, pressed and extracted with warm ammonium oxalate solution. From this the pectinogen was obtained by the method already described. The greater part was, however, obtained from the residue of the apples remaining after expression of the juice, by the method employed in the extraction of pectinogen from turnips. The total yield from apples was very small.

Preparation from rhubarb stem. 16 kilos of rhubarb stem were beaten into fibre by a wooden mallet and the disintegrated mass was pressed in a tincture press. The residue was treated with 4·5 litres of acetone, and then pressed again, first in a tincture and afterwards in a Buchner press. The material thus obtained was dried in an air current at 37° and then ground. The method of preparation of pectinogen from the powder obtained in this way was similar to that employed in the case of turnips. Yield, about 25 g. of air-dried pectinogen, which was of brownish colour.

PROPERTIES OF PECTINOGEN.

Pectinogen is precipitated from aqueous solution by alcohol in the form of a very bulky gelatinous mass, which after treatment with absolute alcohol and drying in air, is converted into a granular powder. It is generally somewhat coloured, the tint depending on the source from which it has been prepared; the product obtained from turnips is nearly white. It dissolves in water somewhat slowly, as a considerable amount of imbibition of fluid must occur before dispersion takes place. It forms viscous, somewhat opalescent solutions which are distinctly acid to phenolphthalein.

0·3 g. of air-dried pectinogen from turnip required 13·3 cc. N/10 acid for neutralisation. (The pectinogen was in this case dissolved in excess of alkali and the excess was titrated back.)

One mol of acid was therefore equivalent to 225 g. of air-dried pectinogen.

A solution of pectinogen made neutral to phenolphthalein by caustic alkalis yields no precipitate on the addition of either dilute acid or a dilute solution of a calcium salt, and in this respect differs from pectin into which it is so readily converted by the method described below.

No method has yet been discovered for preparing pectinogen in a pure state. The product examined was generally obtained from the crude pectinogen by dissolving it in water to form a solution of about 3 %, centrifuging, reprecipitating from this by alcohol, and washing with alcohol of graded strengths up to absolute, in which it was allowed to stand for some time. Specimens obtained in this way generally contained about 3 % of ash after drying *in vacuo* at 100° over phosphorus pentoxide. All specimens used for analysis both of pectinogen and pectin were dried in this manner. Two samples, one from turnips and the other from apples, yielded the following numbers on analysis:

0·2348 g. turnip pectinogen,	0·1129 g. H_2O,	0·3458 g. CO_2,	0·0081 g. ash
0·1420 g. apple „	0·0729 „	0·2153 „	0·0042 „

	H	C	O	Ash
Turnip pectinogen	5·53	41·60	52·87	3·33
Apple „	5·88	42·61	51·51	2·96

The H, C and O numbers are calculated for the ash-free product. They do not differ very markedly from those obtained for pectin, which, there is reason to believe, was obtained in a purer state.

THE CONVERSION OF PECTINOGEN INTO PECTIN.

As mentioned above, pectinogen rapidly undergoes a change when it is kept in alkaline solution at room temperature. A freshly prepared neutral solution gives a precipitate with neither acid nor a dilute solution of a calcium salt. If however it is made alkaline, a change is indicated by the fact that both the above-mentioned reagents will after a short time yield bulky gelatinous precipitates when added to the solution. A gelatinous precipitate is also formed when a pectinogen solution is allowed to stand in the presence of excess of lime-water. There is little doubt that pectinogen is rapidly converted by alkalis into another acid, pectin, which is insoluble in water and gives an insoluble calcium salt. The rate of change from pectinogen into pectin is very rapid and is nearly complete in a few minutes in the presence of normal sodium hydroxide at room temperature. The pectin can then be precipitated as a gel on the addition of acids. It was generally prepared from a 2 % solution of pectinogen which was made distinctly alkaline and then allowed to stand over night at room temperature. From this solution the pectin was precipitated by the addition of an equal volume of concentrated hydrochloric acid, which was used rather than dilute acid as it dissolved out the greater part of the constituents of the ash (see analyses below). The gelatinous precipitate was filtered on to a Buchner funnel by a pump, washed with water till the washings were free from chlorine, and then with alcohol, and finally it was allowed to stand over night under absolute alcohol. It was dried *in vacuo* at 100° over phosphorus pentoxide before being submitted to analysis. It is soluble in alkali and is reprecipitated from the alkaline solution on addition of acids. The yield of pectin was generally about 60 % of the amount of pectinogen taken, but it was found that it is appreciably soluble in strong hydrochloric acid solution. Detailed research, which need not be described here, failed to reveal any other product than pectin, when pectinogen is acted upon by alkalis under the conditions described.

Pectinogen solutions also yield a gel (presumably also pectin) when allowed to stand with concentrated hydrochloric acid at room temperature. The rate of change is however much slower than that which takes place in alkaline solution.

Pectin is soluble in alkalis, and it was found that 0·3 g. of substance dried *in vacuo* at 100° over phosphorus pentoxide neutralised 15·6 cc. N/10 sodium hydroxide. It is not possible from this result, taken in conjunction with the numbers obtained from combustion, to draw any conclusions as to the basicity of pectin, owing to the probability of lactone formation. This point will form the subject of subsequent researches.

Analyses.

Turnip pectin:			
*0·1857 g.	0·0812 g. H_2O,	0·2793 g. CO_2,	0·0040 g. ash
0·2026 ,,	0·0948 ,,	0·3112 ,,	0·0005 ,,
Strawberry pectin:			
*0·1677 g.	0·0770 g. H_2O,	0·2545 g. CO_2,	0·0022 g. ash
*0·1518 ,,	0·0693 ,,	0·2294 ,,	0·0020 ,,
0·1942 ,,	0·0890 ,,	0·2963 ,,	0·0004 ,,
0·1764 ,,	0·0807 ,,	0·2693 ,,	0·0004 ,,
Rhubarb pectin:			
0·1834 g.	0·0892 g. H_2O,	0·2813 g. CO_2,	0·0010 g. ash
0·1842 ,,	——	0·2815 ,,	0·0009 ,,
0·1860 ,,	0·0864 ,,	0·2857 ,,	0·0010 ,,
Apple pectin:			
0·2078 g.	0·0966 g. H_2O,	0·3143 g. CO_2,	0·0012 g. ash
0·1678 ,,	0·0726 ,,	0·2536 ,,	0·0009 ,,

Percentages.

	C	H	O	Ash
Turnip	41·92	4·97	53·11	2·15 *
	41·99	5·21	52·80	0·25
Strawberry	41·94	5·17	52·89	1·31 *
	41·95	5·14	52·91	1·32 *
	41·70	5·10	53·20	0·21
	41·73	5·10	53·17	0·22
Rhubarb	42·06	5·43	52·51	0·54
	41·88	—	—	0·49
	42·12	5·19	52·69	0·54
Apple	41·49	5·18	53·33	0·58
	41·44	4·83	53·73	0·54

The analyses marked with an asterisk were made from samples of pectin which were precipitated from alkaline solution by dilute acid; it will be noticed that in all these cases the ash content is high. The carbon and hydrogen numbers are all calculated on the ash-free product.

As the mean of the above numbers excluding those of the apple pectin, the percentages found are C = 41·93, H = 5·17, O = 52·90. These are in agreement with the formula $C_{17}H_{24}O_{16}$, which requires C = 42·15, H = 4·96, O = 52·89. The numbers for carbon obtained in the pectin sample from apple are a little too low (about 0·5 %), but the other numbers agree in a most satisfactory way with the suggested formula.

Pectin from all sources is soluble in alkali and if strong sodium hydroxide is added to such a solution, the sodium salt is precipitated in the form of a gel. The neutral solution of pectin gives a gelatinous precipitate with calcium chloride.

ESTIMATION OF THE PENTOSE GROUP IN PECTINOGEN.

Pectin itself is insoluble in acids and on heating with them readily undergoes charring. For this reason, the estimation of the pentose groups was carried out with the pectinogen, which is soluble in water, although no method has yet been discovered for purifying the crude product. The specimens employed were dried *in vacuo* at 100° over phosphorus pentoxide, after they had been precipitated from aqueous solution which had been centrifugalised. Both the turnip and rhubarb pectinogens contained 3·4 % of ash. They were heated with hydrochloric acid and the furfural which was evolved was estimated by the Tollens phloroglucinol method. The percentage is calculated on the weight of the ash-free product.

I. 0·3122 g. turnip pectinogen, 0·1114 g. phloroglucinide = 0·0605 g. furfural
II. 0·2935 „ „ 0·1086 „ = 0·0590 „
III. 0·2784 rhubarb „ 0·0912 „ = 0·0500 „
Percentage of furfural from turnips = (I) 20·1 and (II) 20·8
„ „ „ rhubarb = (III) 18·6.

A substance of the formula $C_{17}H_{24}O_{16}$ should yield 19·8 furfural, supposing it to contain one pentose group. As these estimations were carried out with crude pectinogens, the results are as near as could be expected and form strong evidence that each $C_{17}H_{24}O_{16}$ complex contains one pentose group.

SUMMARY.

(1) A method is described for the preparation of the pectic substances of plants, in which the essential operation is the extraction of the residue, obtained after the expression of the juice, with a warm 0·5 % ammonium oxalate solution.

(2) A substance of acidic character is thereby obtained, which is soluble in water and is designated *pectinogen.*

(3) This on keeping in alkaline solution at room temperature readily undergoes conversion into another substance, also of acidic character, which is precipitated from its alkaline solution by acids as a gel insoluble in water. To this substance the name *pectin* has been given. It differs from pectinogen in that it is insoluble in water, and that the solution of its sodium salt gives a gelatinous precipitate with calcium chloride. A solution of pectinogen also gives a gel when allowed to stand at room temperature with an excess of calcium hydroxide solution. In this case, presumably a calcium salt of pectin is obtained. [See however Haynes, 1914]

(4) Pectins from turnips, strawberries, rhubarb stem and apples have been prepared. The analyses agree well with the formula $C_{17}H_{24}O_{16}$. Only in the case of the apple pectin were numbers for the carbon obtained which were slightly too low.

(5) Pectinogen on distillation with hydrochloric acid yields furfural in such a quantity as to indicate that one pentose group is contained in each complex of seventeen carbon atoms.

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XLIV. NOTE ON THE COLOURING MATTER OF RED TORULAE.

By ALFRED CHASTON CHAPMAN.

(*Received November 15th, 1916.*)

Some years ago I undertook in conjunction with Mr F. G. S. Baker, an investigation of the red torulae with the object of isolating as many distinct species as possible, and of obtaining results which might serve as a basis of classification. In connection with this work, during which six different species were obtained and studied, I commenced a subsidiary investigation of the red colouring matter present in these organisms.

One of the six species of torulae above referred to was grown on large plates containing wort-agar. When a sufficient quantity had accumulated the growth was scraped off, care being taken to remove as little of the agar surface as possible. It was then dried at a low temperature and was thoroughly ground with fine sand. The resulting material was then extracted with various solvents, chloroform and carbon disulphide giving the best results. In the case of both these solvents deep red solutions were obtained, which on cautious evaporation gave a coloured residue containing fat and probably some phytosterol. With the small quantities obtained the separation of these fatty substances was found to be impossible, and consequently no crystals could be got. It was found at the commencement of the work that the solution in chloroform when warmed and exposed to the light quickly became colourless, a fact which suggested that the colouring matter might be allied to carrotene. The extracted substance behaved like carrotene in certain other respects, being practically insoluble in water, and dissolving in concentrated sulphuric acid with a deep blue colour. About the time the work had reached this stage, Schimon presented to the University of Munich, a dissertation dealing with certain red coloured lower fungi. He appears to have obtained the same general reactions for the extracted colouring matter, and concluded that it was identical with carrotene. Prior to the publication of this dissertation, I had commenced a study of the absorption spectrum of the colouring matter, with the object of comparing it with that of carrotene, and I observed that the two were not by any means the same. The examination was first made in carbon disulphide solution, with a glass prism, and the spectra were photographed, using Wratton and Wainwright's "Panchromatic" plates. In the case of the colouring matter from the torula, the light passed between wave lengths 5900 to about 7000, whilst in the case of carrotene, a much larger amount of the yellow portion of the spectrum was visible, the range of wave lengths being from about 5500 to 7000. In view of these results, I decided to make an examination of the colouring matter with a quartz prism, so as to obtain some information in regard to the far violet. The source of light used consisted of a Nernst lamp, and the solution of the colouring matter was contained in a Baly tube permitting of a progressive increase in the thickness of the solution examined without changing the containing vessel. For each exposure the position of the sodium lines was recorded by means of a small reflecting prism attached to the instrument, and on each plate the copper arc was photographed as an additional aid to fixing the position of the bands. Solutions of pure carrotene (prepared from carrots) in carbon disulphide were compared side by side with corresponding solutions of the torula colouring matter. In both cases observations were made in thicknesses 1, 2, 3, 4, 5, 6, 8, 10, 13, 16, 20, 25, 32, 40, 50, and 63 millimetres. The strength of the carrotene solution was 5·3 milligrams per litre, and the solution of the torula colouring matter was made of such a strength that the intensity of the absorption bands was comparable with that of the carrotene bands. Wratton and Wainwright's "Allochrome" plates were used, as these are insensitive to red light of greater wave length than about 5890. These, however, show a thin absorption band in the green, due to the colouring matter used in the preparation of the plates. "Panchromatic" plates were found to be less suitable for the purpose. The resulting spectrographs were found to differ very considerably and in a manner which might have been anticipated from a consideration of the absorption spectra as observed with the glass prism. It was found that in the case of the colouring matter of the torula, practically the whole of the yellow is cut off in solutions of sufficient concentration, whereas even in very much stronger solutions of carrotene the whole of the yellow passes. Moreover the absorption band in the blue is much wider in the case of carrotene than in the case of the torula colouring matter.

It is clear from these observations, either that the colouring matter of the torula consists of some other substance than carrotene, or that it consists of a mixture of carrotene with some other colouring matter. The solubility of the torula colouring matter in light petroleum appears to indicate that it does not belong to the xanthophyll group.

My best thanks are due to Dr Monier-Williams and to my assistant, Mr Frederick T. Harry, for valuable help in connection with the preparation of the spectrographs.

XLV. FEEDING EXPERIMENTS WITH DEFICIENCIES IN THE AMINO-ACID SUPPLY: ARGININE AND HISTIDINE AS POSSIBLE PRECURSORS OF PURINES.

By HAROLD ACKROYD and FREDERICK GOWLAND HOPKINS[1].

From the Institute for the Study of Animal Nutrition, Department of Agriculture, and from the Biochemical Department, Cambridge.

(*Received* 19*th December*, *1916*.)

We have abundant evidence to show that purine derivatives can be synthesised in the animal body when no preformed purine material is present in the diet, but the chemical events which are involved in the formation of tissue purines are yet unknown. It is of course possible that the synthesis of the purine ring may start at a low level from simple and remote precursors. Attention has been fixed for instance upon the interesting observation made several years ago by Knoop and Windaus [1905] who showed that 5-methyl-iminazole is formed when a solution of dextrose is exposed to sunlight in presence of the strongly dissociated compound $Zn(OH)_2, 4NH_3$. We have here in effect the formation of the iminazole ring, an essential part of the purine structure, by the action of ammonia upon sugar. That this reaction is related to the chemical mechanism by which histidine and purines are formed in plants is highly probable. In the case of the animal, synthesis of such a type is perhaps less likely. Nevertheless the observations of Cathcart [1909], of Graham and Poulton [1913], and of Umeda [1915] have shown that when the diet is rich in carbohydrates the excretion of endogenous purines is greater than when fat predominates, yielding the suggestion that the former may be employed in synthesis.

It is by no means established however that such a synthesis from carbohydrate derivatives and unknown nitrogenous substances is the sole or chief origin for purines. We are at any rate justified in looking first to the food for a supply of more immediate precursors.

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[1] Several of the experiments described in this paper were made in 1914, the rest in 1915. My colleague has been long at the front, and in writing the paper I have been unable to consult him. He has had moreover no opportunity of reviewing the experimental results as a whole. If therefore it be held that the conclusions are not warranted by the facts I am alone responsible. F. G. H.

When some structural relation is evident between a food constituent and a tissue constituent it is justifiable to suspect that the former, and not some wholly unrelated substance, provides the immediate raw material for the manufacture of the latter. The recognition of such a chemical relation should, at any rate, lead to an experimental enquiry into the possibility of a metabolic relation.

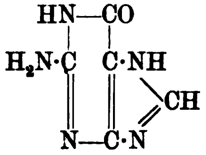
The fact that histidine alone among the amino-acids from protein has a chemical connection with purines in that its molecular structure comprises the iminazole ring has led others to suppose that histidine might be the precursor of purines in metabolism. Abderhalden and Einbeck [1909] tested the point experimentally, but obtained negative results. In their experiments the administration of histidine produced no definite increase in the excretion of allantoin. The experiments were repeated later in conjunction with Julius Schmid [1910]. In the later work a starving dog was given on two occasions an isolated dose of 10 g. of histidine hydrochloride but no increase of allantoin followed.

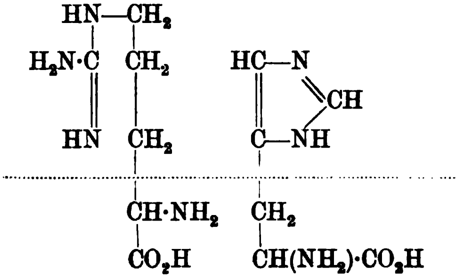
We venture to think however that these observers may not have secured the conditions necessary for properly testing the matter.

When an animal is in a state of full nutrition it does not follow that such a process as the synthesis of the purine ring would necessarily be much accelerated or increased by mere increase in the supply of its raw material. The accepted distinction between endogenous and exogenous metabolism and the recognised relative constancy of the former could scarcely hold were this the case. We know, it is true, that a large increase of pure protein in the diet does affect purine metabolism; but an individual amino-acid fed in excess of the immediate current needs of the tissues, as when it is added to an already efficient dietary, will almost certainly be rapidly broken down on more direct lines, even if it be a normal precursor of the purine (or other) synthesis in the body. We doubt, further, whether the administration of large isolated doses to a starving animal offers the best method for determining whether or not a substance suffers some special fate in metabolism. Doubtless synthesis of such essential tissue constituents as the purines continues during starvation, at the expense—as we are entitled to believe—of protein materials liberated by autolysis of the less essential organs. When however an excess of a single amino-acid enters the circulation of a starving animal in a single isolated dose it may well almost completely escape such special utilisation. It appears suddenly in excess of current needs, and, because the processes of deamination and direct oxidation are always in action, it will almost certainly survive for but a short period as available material for synthesis. The increase in its concentration may momentarily accelerate the special reaction concerned, but the possible increase of reaction velocity in the tissue element is naturally limited, and the survival within the tissue cells of the abnormally increased amino-acid will probably be too short to affect appreciably the day's yield of a synthetic product. Much more satisfactory at any rate would seem to be experiments in which an animal, of which the normal metabolism is known, is first deprived entirely for considerable periods of the substance in question, and deprived of it alone. The effect of its absence being noted the substance can then be restored to the diet in normal amount and given continuously, when the results of its restoration can again be followed for considerable periods.

In the experiments to be described in this paper advantage was taken of the fact that from the complete mixture of amino-acids obtained upon hydrolysis of a typical protein, arginine and histidine can be efficiently removed by the well-known method of Kossel and Kutscher. Purine metabolism can thus be studied upon a dietary from which these two diamino-acids are absent and the effect of the deficiency as well as the result of restoring either or both can be observed.

The original incentive of the present research was, as a matter of fact, the idea that arginine and histidine might function together as precursors of the purine ring in metabolism, on some such lines as the following comparison of the structure of guanine with that of the two diamino-acids might suggest





Our original intention therefore was to test a possibility somewhat different from that which led to Abderhalden and Einbeck's experiments. During the progress of our observations however evidence was obtained which seems to show that in metabolism arginine and histidine are within certain limits equivalent to one another; equivalent probably because each can be converted into the other, just as phenylalanine can, up to a point at least, function in the place of tyrosine [Totani 1916] because in the body it can be converted into the latter (Embden). This circumstance removes some of the significance from the question as to whether both, rather than one only, of these two diamino-acids are concerned in the formation of the purine ring. The point however receives some further attention later on in this paper.

In most of our experiments, after a preliminary period during which a complete amino-acid mixture was administered, arginine and histidine were for some weeks removed from the diet, and then, in a third period, both were restored. The allantoin excretion was followed through each of these three periods.

In our opinion the results obtained offer good grounds for the belief that the molecules of arginine and histidine are as a matter of fact the most readily available raw material for the synthesis of purines in the body.

METHODS.

The experiments were all made upon young growing rats. The rat when it has reached a weight of 80 to 100 g. continues to grow satisfactorily and for long periods on a diet which contains all its nitrogen in the form of a proper mixture of free amino-acids. It is certain however that successful feeding experiments when they involve the administration of abnormal diet can only be carried out on rats when care has been exercised in the choice of stock and when attention is given to the general care of the animals. Some stocks are more prone to disease, and especially to intestinal disorders, than are others, and, if such vulnerable animals are used, intercurrent disease may spoil a prolonged experiment just when it has reached its most interesting period. It is equally important that the experimental animals should be kept in healthy surroundings and at a uniform temperature. The cages should be kept clean and feeding should be carried out at regular intervals.

Admirably suited as is the rat for studies on growth it offers obvious difficulties when quantitative observations have to be made upon the urine.

The amount of urine is of course small and the proclivity of the rat when eating to hold food in its forepaws tends to scattering of the food and, in any ordinary collecting apparatus, to a contamination of the urine with the food.

The cages and apparatus used have been gradually modified by ourselves and by other workers in the laboratory and in their final form, as employed in the present research almost completely obviate any difficulty in collecting the urine quantitatively and with freedom from contamination. The photographs illustrate the apparatus as used (Figs. 1 and 2). The cage itself is made of zinc. It is circular with a diameter of 10 inches, and a depth of 5½ inches, and has a lid of perforated metal. The bottom consists of concentric circles of wire ½ inch apart, held together by radial wires. The food and water are placed in glass vessels which fit into the vertical part of the metal side tubes. The tubes have a diameter of 2¼ inches. When the rat has to obtain its food under the conditions imposed by the tubes it seems unable to carry portions from the trough into the cage. This result

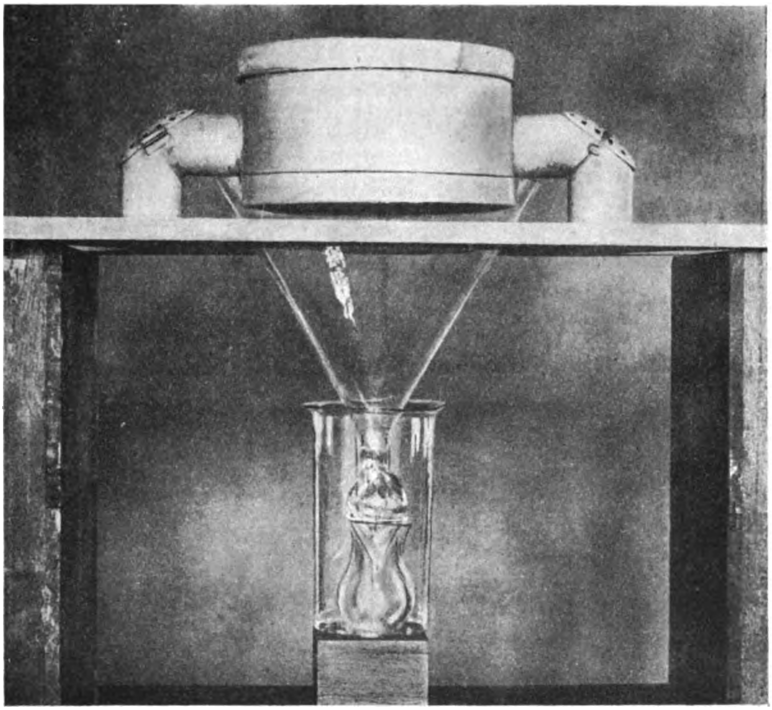


Fig. 1.

is more fully secured when the animal is wholly prevented from turning round while eating from the food-trough. For this reason inner tubes are provided, tapering somewhat towards their distal end. The diameter of these is adjusted to the size of the individual animals under experiment, and they are provided with radial flanges which slide tightly into the outer tube. Other details will be clear from the illustrations.

In our experiments no bedding was provided, as any material used is liable to soak up urine. The absence of bedding makes it important to maintain the cages at a uniform temperature of about 65° F., and it is best that, as in our experiments, two animals should be fed in each cage. All our urine analyses represent the mean of the excretion of two rats.

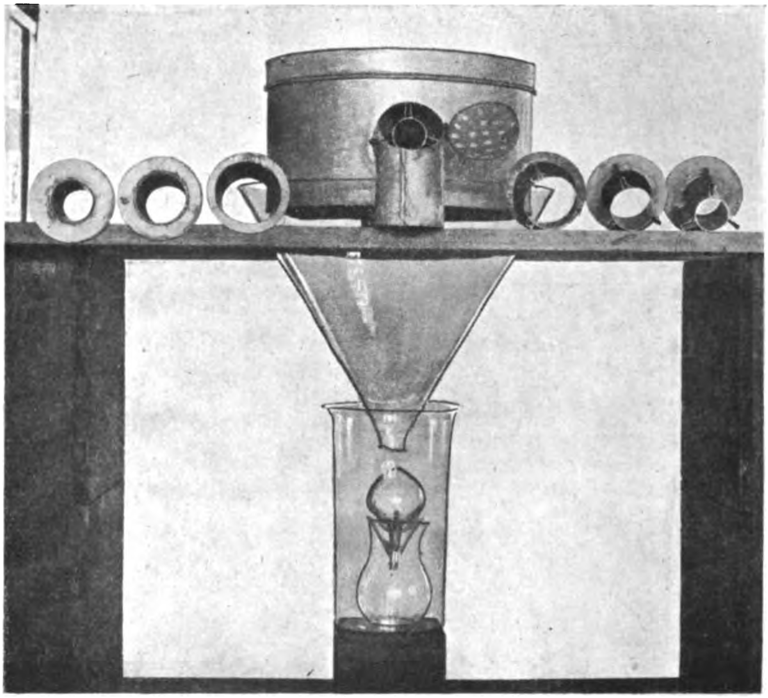


Fig. 2.

For the collection of the urine we employed with slight modifications the apparatus first suggested by Paine and used by him at the Cancer Hospital, Fulham (Fig. 3). The metal cage stands within the upper part of a large glass funnel of somewhat larger diameter than its own. The short neck of the funnel reaches to within about ¾ inch of a glass globe.

This has a diameter of 2¼ inches and is provided with glass supports which allow it to stand in the neck of a small collecting vessel without touching the latter. An intermediary funnel as shown in the photographs may be used if necessary. The urine drops on to the globe without splashing and trickles over its surface into the collecting vessel whereas faeces and food particles are deflected and fall into an outer vessel. We have found it of advantage to give the glass globe a somewhat conical form as shown. The neck of the collecting vessel should have a diameter somewhat less than that of the globe.

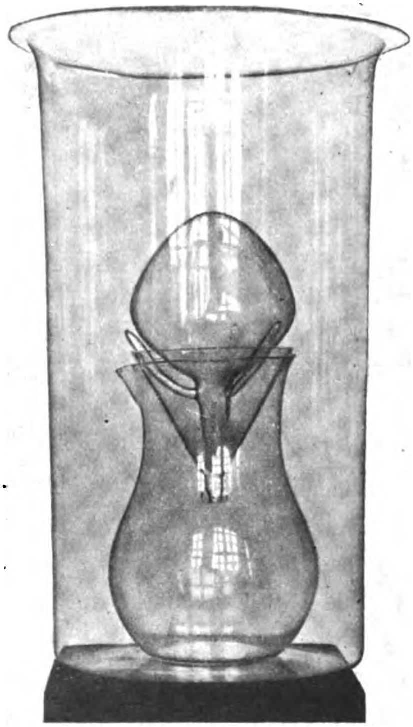


Fig. 3.

In each experiment the analyses were made upon the excretion of successive weeks. Shorter periods are unsatisfactory as their proper demarcation becomes impossible. The funnel in which the cage is placed was washed into the collecting vessel two or three times during the course of each day, small quantities of warm water being used. The urine and washings were transferred at frequent intervals to a stoppered bottle containing chloroform, the whole mixture being kept definitely acid throughout; acetic acid being added when necessary. During the week's collection the material was kept in an ice chest. At the close of the week it was made up to a standard volume for analysis.

For estimation of the allantoin Wiechowski's method was used. We first employed the method as originally described [1908] while in some of our later experiments the more recent modifications were used [Wiechowski, 1913]. In the case of rats' urine however we recommend the preliminary removal of ammonia by evaporation with magnesia and extraction with alcohol whatever modification of the process may be subsequently adopted.

As is well known the final stage of Wiechowski's process is reached either by determining the nitrogen in the mercury-allantoin precipitate, or by actually weighing the allantoin after its liberation from this compound. Wiechowski himself and especially Abderhalden and Einbeck look upon the latter procedure as the more trustworthy. This is not altogether in accord with our experience when working with diets containing free amino-acids. Sometimes a small amount of a syrupy non-nitrogenous substance is then apt to be present when the solutions are evaporated for the crystallisation of the allantoin. This however can be removed by the use of small quantities of ice-cold absolute alcohol. In several of our experiments both methods were used in every determination, and in the great majority of cases the agreement was exceedingly good. We finally came to rely with confidence upon Kjeldahl determinations of the nitrogen in the mercury precipitate. Although some of our experiments were made before the appearance of the paper by Givens [1914], in which he gives the experience of the Cornell laboratories bearing upon the conditions necessary for the stability of allantoin and other points of importance, we had fortunately observed just those precautions (in the preservation of the urine, etc.) which the paper indicates as necessary. Wiechowski's method is long and troublesome, and at no stage can it be entrusted to inexperienced hands. Its complexity limits the number of estimations possible for the individual worker and has prevented us from giving our results the statistical character for which feeding experiments upon small animals like rats otherwise afford the opportunity. As will be seen however our conclusions are based upon results got from a considerable number of individuals. We had hoped to carry out observations with the use of the method for estimating allantoin which has been proposed by Plimmer and Skelton [1914], but our leisure failed. The fault of that method lies in the fact that the allantoin nitrogen is determined by difference. Errors accumulate in estimating what is, after all, only a very small fraction of the total nitrogen. It is nevertheless very desirable that the method should receive full trial. In some experiments the total purine nitrogen of the urine was determined by the silver method of Camerer-Arnstein. Total nitrogen was estimated as usual by the Kjeldahl method.

THE DIETS EMPLOYED.

For experiments of this kind the standard control diet is prepared in the following manner (F. G. H.). Caseinogen, or better, a mixture of equal parts of caseinogen and lactalbumin is hydrolysed by boiling with 25 per cent. sulphuric acid for 20 to 30 hours. The sulphuric acid is quantitatively removed, and the fluid taken down to a syrup. At this stage a weighed quantity of potato starch, somewhat less than that meant to be present in the final diet is thoroughly incorporated with the syrup, and the resulting mixture spread out on a glass plate and allowed to dry in the air for two days. Tryptophane equal to 2 % of the protein originally taken for hydrolysis, and cystine equal to 0·5—1·0 %, are now added and thoroughly mixed with the whole. From this mixture, in which the nitrogen is determined, diets containing varying amounts of fat, additional starch, sugar, and mineral salts can be prepared with any desired nitrogenous content. Upon such diets well selected rats, if otherwise properly cared for, will show satisfactory, if not normal, growth, and will long remain in health. The effects of a tryptophane deficiency can be shown with great ease by refraining from adding that amino-acid to the hydrolysis mixture. Tryptophane certainly appears to be the only protein constituent of nutritive importance which is destroyed by acid hydrolysis, as the favourable results of its addition show. The addition of cystine is perhaps only of importance when the diet is given a low nitrogen content.

When in the present research the animals were to be fed in the absence of either arginine or histidine, or of both, the sulphuric acid of the original hydrolysis was reduced to about 1 %, and the solution of amino-acids well diluted (8 litres for 500 g. of original protein). It was kept at 40° and mixed with silver sulphate until sufficient was present to combine with all arginine and histidine (coloured precipitate on the addition of $Ba(OH)_2$). When working on a large scale this stage of Kossel and Kutscher's process occupies much time, but it is very essential that it should be properly carried out, and a full excess of silver added. The fluid was next saturated, while still at 40°, with powdered barium hydroxide. After cooling, the precipitate was filtered off at the pump and very thoroughly washed. From the bulky filtrate every trace of silver and barium was removed, and the whole evaporated to a syrup. In the preparation of the special diet this syrup was treated exactly as described above for the complete hydrolysis mixture. Tryptophane and crystine were added at the proper stage, and if only one of the two diamino-acids was required to be absent a supply of the other was restored to the mixture. When the effect of restoring both to the food was under study the animals were not simply put upon a total hydrolysis mixture separately prepared, but arginine and histidine were restored to a mixture which had previously undergone treatment by the silver process, the results being thus more strictly comparable.

The silver method seems to remove both arginine and histidine with great completeness. If traces are left behind, and it is very difficult to disprove this possibility, the fact would not affect the bearing of our experiments, which were all comparative and controlled. The presence of traces in the diet of the experimental periods would tell against, rather than in favour of, our main conclusions.

The following example will illustrate the composition of the dietaries as fed:

Amino-acids from	200 g. of caseinogen
Potato Starch	250 ,,
Cane Sugar	125 ,,
Lard	45 ,,
Butter	45 ,,
[1]Ash from equal weights of Oats and Dog biscuit	21 ,,

This particular mixture contained 3·12 % of nitrogen; less than calculation from the original protein would indicate, but there is always some loss during the manipulation of the hydrolysis mixture. In some cases the diets have contained less nitrogen (down to 2 %) with proportionately more starch and sugar and fat. In each individual experiment the diet was identical throughout except for the qualitative variation in the amino-acid supply. Any change in purine metabolism was therefore independent of changes in the carbohydrates or fats.

The essential need of a vitamine supply comes to light with the greatest possible clearness when synthetic diets are prepared with amino-acids got by acid hydrolysis. Without a suitable addition there is no growth, and but short maintenance, when the assembly of amino-acids is complete. In our experiments this supply was given in the form of a protein-free alcoholic extract of fresh milk solids. Each rat received daily the equivalent of about 5 cc. of milk. This was true of every experiment described. To save printing we have not given the food consumption of our animals in the account of the experiments in the next section. We may state here that it was, throughout, sufficient to supply the energy not only for maintenance but for proper growth. This was true in the case of every experiment of which the protocol is given.

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[1] There is no special reason for employing this particular mineral supply. It was originally used because oats and dog biscuits were the main food of stock rats in the laboratory. The ash has always proved satisfactory and large quantities were originally made so that its use has been continued.

EXPERIMENTAL AND CRITICAL.

Effects upon Nutrition. Before giving the results of our allantoin estimations it will be convenient to deal separately with the effect upon body weight of the various dietetic deficiencies in connection with which we have studied the purine metabolism.

The pronounced effect upon nutrition produced by the withdrawal of tryptophane has, since the publications of Willcock and Hopkins, Osborne and Mendel, Abderhalden, Henriques and Hansen, and others, become well recognised. One of us (H.) has in various connections carried out a large number of experiments in which the nutritive condition of rats with and without tryptophane has been compared. Chart I summarises some of these results so far as the effect upon growth or body weight is concerned. The continuous lines show the effect of first withdrawing and of subsequently restoring tryptophane in the case of three separate rat pairs. The tryptophane was withheld on the 12th day (as shown in chart; the actual preliminary period was longer) of a diet containing a complete mixture of amino-acids. It was restored on the 35th day. The dotted lines show the average rate of growth upon the complete mixture (16 animals), and the average rate of loss in body weight when tryptophane is absent (8 animals). The average growth rate though satisfactory was not normal; but quite normal growth has been obtained in certain individuals when upon the complete mixture (hydrolysis products + tryptophane and a little cystine). On the other hand in a few cases not much more than maintenance without growth was attained [Asayama 1916] but this appears to have been due to deficiency in cystine as the result of prolonged hydrolysis of the protein, or to lack of adjustment in the vitamine supply. These cases were not included in the group yielding the average shown. After short hydrolysis of the protein (6 to 8 hours) maintenance, and some slight growth, has been observed in a few individuals, when no tryptophane was added to the hydrolysis products. Under these circumstances a certain amount of tryptophane escapes destruction and though certainly small it makes itself felt in nutrition. In all the

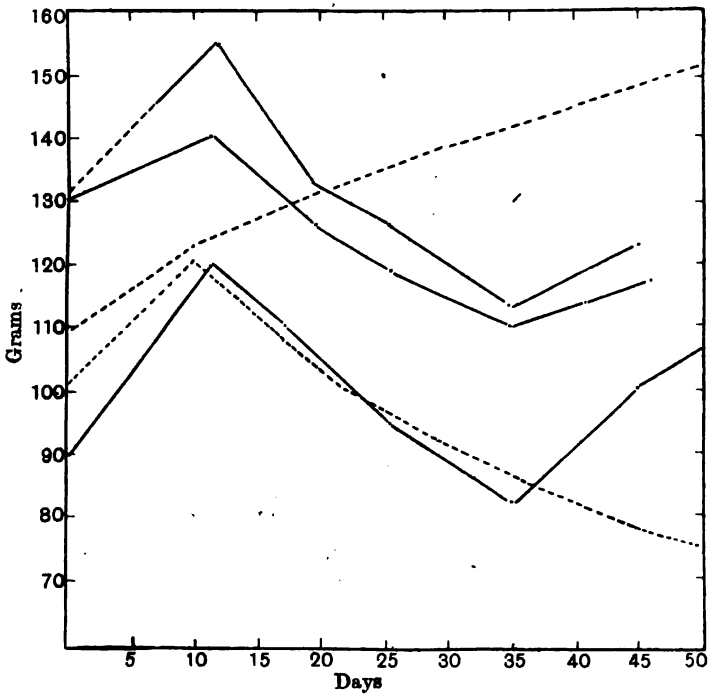


Chart I. Showing the effect upon body weight of withdrawing tryptophane from the diet. Continuous lines show mean weight of two rats in each case. Tryptophane removed on 12th day, restored on 35th day. The ascending dotted line shows average growth of 16 rats on complete amino-acid mixture. Lower dotted line average loss of 8 rats deprived of tryptophane.

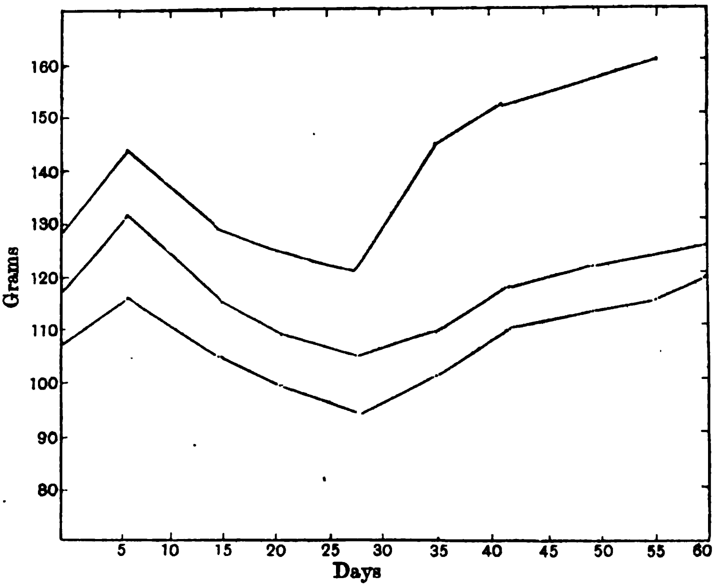


Chart II. Effect of removing arginine and histidine together. Mean weights of rat pairs. Diamino-acids removed on 7th day and restored on 28th day.

experiments yielding the results shown in Chart I the hydrolysis of the protein was continued for 24 hours or more.

When arginine and histidine are together removed from the products of protein hydrolysis the residual amino-acids are unable to support the animal. Chart II shows the effect upon body weight which the removal and restoration of these two diamino-acids respectively produce. When the removal has been as complete as possible the weight of animals taking the diet falls sharply. We have found however that this decline in weight becomes after a time less rapid than in the case of the tryptophane deficiency. There seems to be some adjustment to the lack of arginine and histidine. True, it must not be forgotten that, while prolonged hydrolysis destroys every trace of tryptophane, Kossel and Kutscher's method may leave behind minute amounts of the diamino-acids. The adjustment may be, not to the absence of arginine and histidine, but to the utilisation of a minute amount, sufficient for maintenance at a certain level, though not for growth. We believe

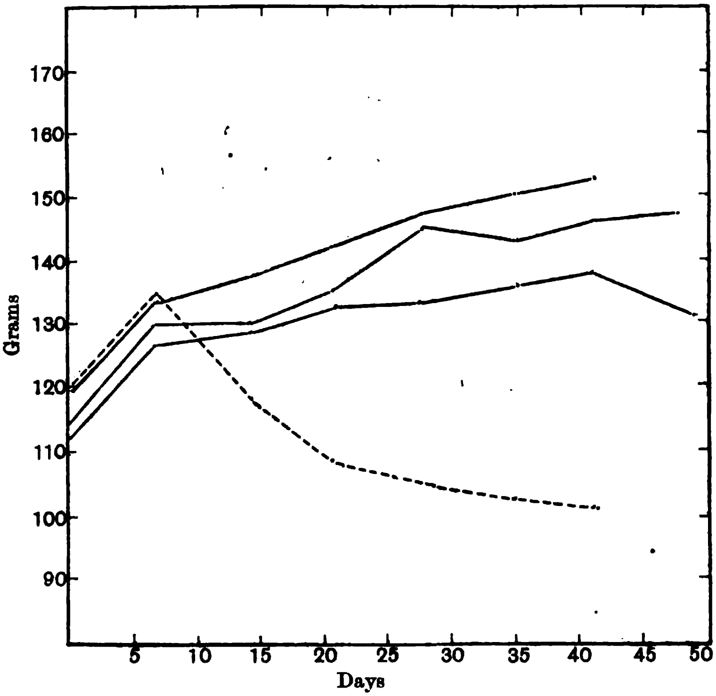


Chart III. Dotted line, body weight changes in the absence of both arginine and histidine. Lowest continuous line shows result when, of the two, only arginine was present. Two upper curves give weights when only histidine was present. Average weights of two rats in each case.

nevertheless that our preparations were free from these substances and that there are as a matter of fact different degrees of urgency in the need of an animal for a supply of this or that individual amino-acid in its food. On this assumption one of us has elsewhere briefly discussed the bearings of the present case [Hopkins, 1916].

The observations now to be mentioned illustrate a somewhat different aspect of the question as to how far the animal can dispense with individual protein units. They appear to us to yield an instance showing that the body can with some efficiency utilise one amino-acid vicariously for another.

The circumstance came to light when to the amino-acid mixture previously deprived of arginine and histidine, one, and only one of these two diamino-acids was restored.

The dotted line of Chart III shows once more the results when both are absent. It exhibits the average weights of two animals which were deprived of both substances for a somewhat longer period than in the earlier experiments (Chart II). In very marked contrast is the result of feeding when either one of the two is present in the diet. The two middle curves of Chart III show the behaviour of rat-pairs when receiving in two strictly comparable experiments either histidine without arginine (upper of these curves) or arginine without histidine (lower curve). There is no loss of weight such as follows when both are absent, but maintenance, and even slight growth. These results were obtained when the amount of arginine or histidine respectively amounted to only 3 % of the total amino-acid mixture. In another experiment (uppermost curve) histidine was added to the extent of rather under 5 % of the whole mixture and appreciable growth followed upon the diet, although arginine was absent. These results receive further attention in a later discussion.

Effects upon Purine Metabolism. We shall now give the protocols of experiments in which the allantoin excretion was determined week by week when arginine and histidine were first removed from, and afterwards restored to, the diet.

It will be seen from colums 4 and 7 of the protocol that in Experiment I the replacement of a bread and milk diet by one containing an amino-acid mixture deprived of the two diamino-acids, led, by the second week of the substitution, to a fall of over 40 % in the allantoin excreted. The excretion rose somewhat in the third week, but during the fourth it was only one half of the amount shown on the normal diet. When, in the sixth week of the experiment, arginine and histidine were restored to the amino-acids of the diet the excretion of allantoin immediately rose, and after three weeks of feeding on the completed mixture it reached nearly 90 % of its original value.

In Experiment II, done under similar conditions, there was a fall of 47 % during the second week of deficiency feeding, and the results, in general, are very similar to those of Experiment I, save that there was a degree of lag in the effects of restoration. In the first week of giving the completed amino-acid mixture the excretion of allantoin remained nearly at its lowest, but it rose rapidly in the two succeeding weeks.

Arginine and Histidine absent from Food.

Experiment I.

Week 1914	Mean weight during week: Rat *A*	Mean weight during week: Rat *B*	Allantoin from two rats per week	Nitrogen per rat per day: Total N	Nitrogen per rat per day: Purine N	Nitrogen per rat per day: Allantoin N	Nitrogen per rat per day: Allantoin N as percentage of total N.	Diet
1 (18. VI.–25. VI.)	124	132	0·458	0·147	0·00065	0·0116	7·8	Bread and Milk
2	116	126	0·296	0·095	0·00044	0·0080	8·4	Amino-acids without arginine or histidine
3	109	116	0·260	0·107	0·0003	0·0065	6·3	,, ,, ,,
4	105	109	0·319	0·116	0·0004	0·0080	6·7	,, ,, ,,
5	102	106	0·228	0·104	0·00023	0·0058	5·6	,, ,, ,,
6	113	114	0·347	0·134	0·00036	0·0090	6·7	Amino-acid + A. and H.
7	121	124	0·347	0·133	0·00034	0·0090	6·7	,, ,, ,,
8	131	131	0·400	0·157	0·00049	0·1010	7·0	,, ,, ,,
9	137	141	0·454	0·199	0·00069	0·1150	5·8	Bread and milk

Experiment II.

Week	Rat *C*	Rat *D*	Allantoin from two rats per week	Total N	Purine N	Allantoin N	Allantoin N as percentage of total N.	Diet
1	146	125	0·575	0·182	0·00063	0·0146	8·0	Bread and milk
2	139	119	0·406	0·105	0·00044	0·0103	9·8	Amino-acids – A. and H.
3	125	112	0·306	0·111	0·00046	0·0077	7·0	,, ,, ,,
4	119	106	0·390	0·142	0·00037	0·0099	7·0	,, ,, ,,
5	120	106	0·310	0·130	0·00039	0·0079	6·1	Amino-acids + A. and H.
6	125	115	0·413	0·119	0·00046	0·0105	8·8	,, ,, ,,
7	131	120	0·400	0·135	0·00053	0·0099	7·3	,, ,, ,,
8	133	127	0·486	0·136	0·00050	0·0123	9·0	,, ,, ,,
9	142	137	0·501	0·167	0·00051	0·0136	8·1	Bread and milk

In the above experiments the food of the first control period was a purine-free, but otherwise normal, dietary (bread and milk). We have found that usually, though not always, a fall from 10 to 15 % in the allantoin seems to follow when the food is changed from bread and milk to a dietary containing even a complete amino-acid mixture. This result does not depend upon any general failure of nutrition, as it may occur when the weight is going up and the total consumption of nitrogen greater than during the bread and milk period. It is not easy to explain. The bread and milk consumed by two rats during a week would—to judge from determinations made by one of us [Ackroyd 1911]—contain enough allantoin to add 10 to 12 mgs. to the week's excretion. It might possibly add more, but not enough to account for the result mentioned. If such views as those of Marěs [1888] are accepted it is possible that because liberated amino-acids instead of intact protein enter the alimentary canal there is less activity of the digestive glands and therefore some lessening of purine metabolism. Possibly the fact that the free acids are absorbed rapidly, rather than gradually as during normal digestion, may affect the efficiency of synthesis in the body. Growth upon the complete amino-acid mixture though satisfactory is seldom of normal velocity.

In any case the circumstance in no way affects the significance of the experimental results. Experiments I and II should be compared with Experiments VI and VII which were done under precisely similar conditions.

In the two experiments which follow a complete amino-acid mixture formed the nitrogen supply of the preliminary, as well as of the final, control period.

It may be further noted that in the first two experiments when arginine and histidine were restored to the diet they were not isolated but were added as recovered together from the silver-barium precipitate of the Kossel and Kutscher process. In the later cases they were added pure, in the form respectively of the nitrate and hydrochloride. Sodium carbonate was simultaneously added in quantity sufficient to correspond with the acid radical of the salt.

In Experiment III in which during the preliminary control period the complete amino-mixture and not bread and milk was given, the urine during the first week of feeding without arginine and histidine was unfortunately lost. That of the second week of the deficiency still contained nearly 85 % of the original output of allantoin. There was apparently therefore a delay in the effect of the deficiency not seen in the first experiments. During the third and fourth weeks however the allantoin fell to 60 % of its original value, and then rose immediately when the arginine and histidine were restored.

In Experiment IV the allantoin was sharply reduced when arginine and histidine were removed from the complete mixture of amino-acids first given. In the second week of the deficiency it had fallen to one half. It rose somewhat in the two succeeding weeks, but in the fourth week it was only 64 % of its original value. On restoration of the missing amino-acids it quickly rose to the original amount.

Although the time incidence of the minimum varied in the above experiments the removal of arginine and histidine from the food never failed to produce a noteworthy decrease in the allantoin excreted.

Arginine and Histidine absent.

Experiment III.

	Mean weight during week		Allantoin from two rats per week	Nitrogen per rat per day				
Week 1915	Rat *E*	Rat *F*		Total N	Purine N	Allantoin N	Allantoin N as percentage of total N	Diet
1 (11. v.–18. v.)	136	125	0·366	0·121	—	0·0092	7·6	Complete amino-acid mixture
2	134	123	—	—	—	—	—	Arginine and histidine absent
3	127	115	0·312	0·120	—	0·0079	6·6	,, ,, ,,
4	126	114	0·237	0·110	—	0·0060	5·5	,, ,, ,,
5	123	110	0·221	0·112	—	0·0058	5·2	,, ,, ,,
6	137	113	0·356	0·150	—	0·0090	6·0	Arginine and histidine restored
7	149	121	0·356	0·147	—	0·0090	6·1	,, ,, ,,
8	155	128	0·343	0·130	—	0·0087	6·7	,, ,, ,,

Experiment IV.

	Rat *G*	Rat *H*						
1	124	115	0·400	0·118	—	0·0101	8·5	Complete amino-acid mixture
2	129	123	0·406	0·115	0·00051	0·0103	9·0	,, ,, ,,
3	120	118	0·301	0·107	0·00039	0·0076	7·1	Arginine and histidine absent
4	113	111	0·210	0·109	—	0·0053	4·8	,, ,, ,,
5	110	106	0·290	0·101	0·00029	0·0073	7·2	,, ,, ,,
6	106	102	0·256	0·098	0·00023	0·0065	6·6	,, ,, ,,
7	112	109	0·374	0·110	—	0·0095	8·6	Arginine and histidine restored
8	119	115	—	0·114	—	—	—	,, ,, ,,
9	126	119	0·405	0·121	0·00057	0·0102	8·4	,, ,, ,,

Experiments V and V*a* show the effects produced upon the allantoin when of the two diamino-acids only one is absent from the food. In the first arginine was present and histidine absent; in the second arginine was alone wanting. In the control periods the animals received the complete amino-acid mixture. In neither case was the fall at any period greater than 17 %. This is in marked contrast with the 40 to 50 % decrease induced by the absence of both substances. We hold that this interesting result supports the evidence from the body weight curves in suggesting that arginine and histidine are essentially equivalent in metabolism.

To control the experiments already described and to decide so far as possible whether the reduction of allantoin represents the failure of a specific synthesis in the body, and is not an indirect phenomenon depending upon the general failure in nutrition, we have studied the effect of other deficiencies.

The effect of removing tryptophane was first investigated. In Experiments VI and VII the diet of the first control period was bread and milk. This was immediately followed by food containing an amino-acid mixture deprived of tryptophane. These two experiments are therefore to be compared with Experiments I and II. In neither do we find the effect upon allantoin produced by the absence of arginine and histidine. In Experiment VI it is true a disturbance in the purine metabolism is shown which it is not easy to explain. After two weeks of the deficiency, showing no appreciable effect upon the allantoin, there was in the third week a sudden rise, and, in the following week what seems to be a compensating fall. The average of the two weeks represents an excretion which was nearly normal.

Histidine or Arginine removed.

Experiment V.

Week	Mean weight during week		Allantoin from two rats per week	Nitrogen per rat per day			Diet
	J_A	K_A		Total N	Allantoin N	Allantoin N as percentage of total N	
1	120	122	0·410	0·170	0·0104	6·1	Total amino-acids
2	120	125	0·390	0·171	0·0099	5·8	Arginine present, histidine absent
3	118	127	0·351	0·152	0·0089	5·8	,, ,, ,,
4	115	127	0·337	0·140	0·0086	6·1	,, ,, ,,
5	114	128	0·343	0·134	0·0087	6·5	,, ,, ,,
6	114	125	0·343	0·130	0·0087	6·7	,, ,, ,,

Experiment V*a*.

Week	J_H	K_H	Allantoin from two rats per week	Total N	Allantoin N	Allantoin N as percentage of total N	Diet
1	135	131	0·448	0·189	0·0106	5·6	Total amino-acids
2	134	130	0·441	0·180	0·0105	5·8	Histidine present, arginine absent
3	137	131	0·400	0·144	0·0099	6·9	,, ,, ,,
4	138	139	0·374	0·137	0·0095	6·9	,, ,, ,,
5	137	138	0·374	0·142	0·0095	6·7	,, ,, ,,
6	134	131	0·356	0·140	0·0090	6·4	,, ,, ,,

In Experiment VII there was no irregularity of this sort. The replacement of bread and milk by an amino-acid diet without tryptophane was associated with a maximal fall —in the second week—of 19 %. In the third week the excretion was only 9 % below that of the bread and milk period. Restoration of tryptophane to the diet produced no rise.

In Experiments VIII and IX the diet of the preliminary control period was one containing the complete amino-acid mixture. They are therefore comparable with Experiments III and IV. The removal of tryptophane produced no effect upon allantoin at all comparable with that seen when arginine and histidine are absent. In certain weeks when upon the deficiency diet the excretion of the animals was above that of the control period.

Tryptophane absent from food.

Experiment VI.

Week 1914	Mean weight during week		Allantoin from two rats per week	Nitrogen per rat per day			Diet
	Rat *M*	Rat *N*		Total N	Allantoin N	Allantoin N as percentage of total N	
1 (4. xi.–13. xi.)	106	98	0·410	0·170	0·0104	6·1	Bread and milk
2	118	105	—	—	—	—	„
3	118	107	0·392	0·198	0·0098	4·9	Amino-acids, tryptophane absent
4	111	100	0·410	0·171	0·0104	6·1	„ „ „
5	103	94	0·536	0·150	0·0136	9·0	„ „ „
6	98	90	0·245	0·126	0·0062	4·9	„ „ „
7	109	100	0·326	0·136	0·0083	6·1	Tryptophane restored
8	129	117	0·380	0·154	0·0096	6·2	„ „
9	135	122	0·406	0·167	0·0103	6·2	Bread and milk

Experiment VII.

	Rat *O*	Rat *P*					
1	107	107	0·393	0·141	0·0101	7·2	Bread and milk
2	107	107	0·351	0·157	0·0089	5·7	Amino-acids without tryptophane
3	102	102	0·335	0·160	0·0085	5·3	„ „ „
4	91	92	0·357	0·153	0·0095	6·2	„ „ „
5	92	95	—	0·130	—	—	Total amino-acids
6	104	98	0·319	0·123	0·0081	6·5	„ „

In Experiment X the periods observed were short. It is of interest however because in it the same rats were fed first without arginine and histidine and then, after an interval with normal feeding, without tryptophane. A marked fall in the allantoin (40 %) occurred under the former conditions, and a much smaller one (10 %) in the latter. In each case the food of the preliminary period was bread and milk.

The difference between the effect of removing tryptophane and that of removing arginine and histidine is throughout the experiments quite unmistakable.

Tryptophane absent.

Experiment VIII.

Week 1915	Mean weight during week Rat *S*	Rat *T*	Allantoin from two rats per week	Nitrogen per rat per day Total N	Allantoin N	Allantoin N as percentage of total N	Diet
1 (10. II.–15. II.)	141	134	—	—	—	—	Total amino-acids
2	152	141	0·319	0·104	0·0081	7·8	,, ,,
3	146	135	0·319	0·179	0·0081	4·5	Tryptophane absent
4	130	122	0·347	0·160	0·0088	5·5	,, ,,
5	118	114	0·300	0·151	0·0076	5·1	,, ,,
6	109	108	0·304	0·130	0·0077	5·9	,, ,,
7	110	108	0·308	0·102	0·0078	7·6	Tryptophane restored
8	118	113	0·276	0·114	0·0070	6·1	,, ,,

Experiment IX.

	Rat *SS*	Rat *TT*					
1	220	225	—	—	—	—	Total amino-acids
2	215	222	0·347	0·276	0·0088	3·2	,, ,,
3	202	207	0·400	0·277	0·0099	3·6	Tryptophane absent
4	189	193	0·343	0·286	0·0087	3·1	,, ,,
5	176	178	0·300	0·196	0·0075	3·8	,, ,,
6	171	172	0·400	0·266	0·0099	3·7	,, ,,
7	181	179	0·356	0·202	0·0090	4·4	Tryptophane restored
8	199	197	0·420	0·322	0·0107	3·3	,, ,,

Experiment X.

Week 1914	Mean weight during week or period Rat *AA*	Rat *BB*	Allantoin from two rats per week	Nitrogen per rat per day Total N	Purine N	Allantoin N	Diet, etc.
1 (6. III.–13. III.)	210	201	0·400	0·200	0·0010	0·0101	Bread and milk Three days on amino-acids minus arg. and hist.
2 (16. III.–23. III.)	182	188	0·239	0·1910	0·0006	0·0060	ib. (week)
3 (30. III.–5. IV.)	187	189	0·325	0·220	0·0008	0·0082	Total amino-acids Rats for three weeks on bread and milk
Ten day periods							
1	242	262	0·512	0·259	0·0010	0·0130	Bread and milk
2	228	251	0·458	0·229	0·0008	0·0116	Amino-acids minus tryptophane
3	213	242	0·458	0·2300	0·0008	0·0116	,, ,, ,,
4	215	244	0·441	0·1700	0·0005	0·0112	Tryptophane restored
5	259	278	0·473	0·225	0·0012	0·0120	Bread and milk

We have in one experiment studied the effects of quite another type of dietetic deficiency upon the allantoin excretion—the lack, namely, of exogenous growth hormones, or vitamines. As this involves nutritive failure of a marked kind the results offer further control of the arginine and histidine results.

Two rats were fed for eight weeks upon a synthetic diet containing purified caseinogen. During the first four weeks each animal received 2 cc. of fresh milk a day [Hopkins 1912]. In the last four weeks this was withheld, and, as the result of the absence of vitamines, the weight of the animals, which had risen during the first period, then fell rapidly, especially in the last two weeks. We give the results of the experiment in brief form.

	With Vitamine supply				Without Vitamine supply			
Week	1	2	3	4	5	6	7	8
Allantoin from two rats per week	0·263	0·319	0·265	0·285	0·294	0·350	0·340	0·296
Total N from two rats per week	2·47	2·83	2·91	—	3·25	3·38	4·08	3·50

The increase in food consumption after the withdrawal of the small dose of milk has been observed before. It was somewhat disconcerting in the present experiment. Nevertheless the first period was one of growth, and the second one of declining weight. We have further proof therefore that the decrease in allantoin due to the withdrawal of arginine and histidine is not due to nutritive failure alone, for in this experiment there was no fall.

It may be asked here how far the excretion of allantoin in the rat changes with increase or decrease of the total nitrogenous metabolism. All that we know concerning purine metabolism in other animals when the diet is purine-free would lead us to expect that there would be no direct proportionality.

In the protocols of our main experiments will be found a column giving the allantoin-nitrogen as a percentage of the total nitrogen. It will be seen that in general the relation is irregular because of disproportionate and independent variations in the two quantities. It may be noted however that the ratio of allantoin-N to total-N always tended to fall markedly as the result of arginine and histidine deficiency.

We carried out one brief experiment intended to bear specially on the relation of allantoin to total nitrogen. A pair of rats was for one week fed upon a diet of bread and water. Then for one week a large proportion of caseinogen was supplied, while in a third week the animals returned to bread and water. The results were as follows:

	1st week	2nd week	3rd week
Allantoin	0·408	0·404	0·465
Total Nitrogen ...	2·26	4·27	2·35
Ratio of A.-N. to T.-N.	1 : 16	1 : 30	1 : 14

In this particular experiment the moderate rise in allantoin which occurred was not seen until the animals were again upon the diet containing the lower proportion of protein. Our main experiments were clearly not of a kind to afford evidence upon the point. The distinction between endogenous and exogenous purine excretion is, however, known to be vaguer than was once thought. A large increase of protein in the food has been shown to increase the output of purines, at least in some animals.

The behaviour of the tissues complicates the phenomena. We may perhaps picture the relations in the following way. Actual starvation increases the excretion of endogenous purines because of tissue breakdown. Once equilibrium is re-established on a proper food supply, the normal activities of the tissue determine and limit the amount of purine production through a somewhat wide range of protein intake; until ultimately, when the intake is large enough, the chemical reactions involved are inevitably accelerated by the continued high concentration of new material in the cell. It is clear that if a particular constituent of the protein molecule rather than any other is concerned in purine synthesis, experiments in which the tissues were at first wholly deprived of that constituent are those most likely to demonstrate the fact.

DISCUSSION OF RESULTS.

Our observations show that the simultaneous removal of arginine and histidine from its food produces a marked decrease in the excretion of allantoin by the rat. The excretion observed when a normal purine-free diet was given, or that upon a diet containing a full assembly of amino-acids, was reduced by some 40 to 50 % during the period (1 to 4 weeks) when the animals were deprived of these two diamino-acids. The actual minimum of allantoin occurred sometimes early and sometimes later, but throughout the period of the deficiency it was always notably below the normal. On restoration of the missing diamino-acids it returned to or approached its original level. These effects seem to be definite and constant.

Since however deprivation of the two diamino-acids produces at the same time a marked effect upon the general nutrition of the animal, involving at first a rapid loss of weight, it is clear that the relation thus brought to light cannot be at once accepted as a proof that arginine and histidine are direct precursors of the purines of the body. The effect of their removal upon the allantoin excretion might be indirect, and a partial expression of the general nutritional failure. We know of no information in the present literature of the subject which could assist in measuring this possibility.

In actual starvation, to judge from Cathcart's observations upon a fasting man [Cathcart 1907], there is first a brief fall and, later, a rise in the purine excretion. The fall in the case studied by Cathcart lasted for three days only. A period of three or four days in the case of a man is out of all proportion small when compared with one of three or four weeks in the case of a rat. The brief fall at the beginning of starvation depends certainly upon factors different from those responsible for our results.

Moreover a qualitative deficiency in the amino-acid supply cannot be compared with actual starvation. The animals in our experiments were receiving an abundant energy supply throughout the periods observed, in spite of the failure in nutrition, and their nitrogenous metabolism was sometimes above the normal. There was no experimental information which could enable us to judge as to what might be the indirect effect if any of a general nutritive condition of this sort upon the special phenomena of purine metabolism.

By far the best method of controlling our results was—it seemed to us—to compare the effects of the deficiency in arginine and histidine with those of another deficiency in the diet, and, especially, of another amino-acid deficiency. We therefore fed rats upon a diet of which the nitrogenous supply was contained in an amino-acid mixture deprived of tryptophane. In the absence of this constituent the disturbance in the nutritional balance and the loss of weight are even more marked than when arginine and histidine are absent. In these control experiments all other factors in the diet, and all the other experimental conditions were made identical with those of the main experiments. The relative proportions of carbohydrate and fat, for instance, were the same. The only variation was in the nature of the amino-acid or acids removed from the food. Such a comparison should eliminate the influence of many unknown or uncertain factors.

Examination of the protocols given in the last section will show that the removal of tryptophane produced no fall in the excretion of allantoin at all comparable with that seen when arginine and histidine are absent. The excretion was somewhat irregular; at times there was a small fall during part of the deficiency period, at other times there was some increase; on one occasion there was a considerable rise during the third week followed by a compensatory fall in the fourth. Taking the results as a whole however the difference between the effects of removing tryptophane and those which follow the removal of arginine and histidine is sharp and unmistakable. In the latter case the decrease in allantoin is constant and of marked degree.

It happens that in the case of the tryptophane deficiency the mean weekly excretion of the animals (17 weekly estimations) was identical with that of animals upon the complete amino-acid mixture (25 estimations) namely 0·135 g. allantoin per rat per week. This may be a coincidence but it is good evidence that there was no decrease of any importance.

In no case, it should be noticed, did we extend our observations concerning the allantoin excretion beyond the fourth week of feeding upon the deficient amino-acid mixture. At this stage and sometimes sooner we restored the missing constituent to the diet in order to control the results. We do not know what may happen to the allantoin at later periods. For the specific purpose of the research it seemed better to compare the effect of the different diets upon purine metabolism when the nutritional failure was as yet not too marked. Premortal changes might introduce complications and obscure the result.

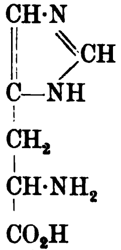
A dietetic deficiency of quite another kind, the absence namely of growth hormones or vitamines, produced, in the one experiment carried out to test the matter, no fall in the excretion of allantoin, although the animals were losing weight as fast as those fed without the diamino-acids.

All our results therefore point to the probability that there is a specific metabolic relation between arginine and histidine and the purines of the body.

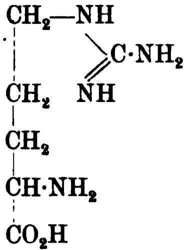
It was explained in the introductory paragraphs of this paper that we began our experiments in the belief that the molecules of arginine and histidine might prove to be used conjointly in metabolism as precursors of the purine ring. Our observations upon body weight, and, no less, others upon allantoin excretion seem to show clearly however that either one of these two diamino-acids can with a considerable degree of efficiency subserve the functions of the other. When both are removed from the diet the nutritive failure and the fall in allantoin excretion are unmistakable. When either alone is restored in sufficient amount there is maintenance and even growth, while no marked fall in allantoin is observed.

On our own view this is because each of these protein derivatives is, in metabolism, capable of being converted into the other, and is so converted when the supply of both is not harmoniously adjusted for the needs of the animal.

The chemical relations which justify this view are familiar, and become clear when the structural formulae are properly compared.



Histidine



Arginine

If arginine and histidine are, in the sense just discussed, to a large extent, equivalent in metabolism our original view that the molecules of both, rather than the molecule of histidine alone, are concerned in the synthesis of the purine ring loses some of its point. That the presence of both in the food yields the optimum conditions for purine synthesis seems nevertheless to be probable.

SUMMARY.

(1) When arginine and histidine are together removed from the diet of rats which have been previously growing on a complete amino-acid mixture there is a rapid loss of body weight. This is promptly succeeded by renewed growth when the missing diamino-acids are restored to the diet.

(2) When arginine alone, or histidine alone, is restored to the food there is no loss of weight and there may even be growth. Nutritional equilibrium is possible in the absence of one of these related protein constituents though not in the absence of both. It is suggested that this is because each one of them can, in metabolism, be converted into the other.

(3) When arginine and histidine are both removed from the food the amount of allantoin in the urine is much decreased. When they are replaced the excretion returns to the normal. The decrease is very much less when either one of these diamino-acids is present alone.

(4) No decrease of allantoin excretion occurs when tryptophane is removed from the food, though nutritional failure is even greater than when arginine and histidine are withheld. No decrease was observed when animals were losing weight as the result of the absence of a vitamine supply.

(5) It is suggested therefore that arginine and histidine play a special part in purine metabolism, probably constituting the raw material (or the most readily available raw material) for the synthesis of the purine ring in the animal body.

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